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THE METABOLISM OF YEAST SPORULATION

I. EFFECT OF CERTAIN METABOLITES AND INHIBITORS[1]

BY J. J. MILLER AND CLARA HALPERN

Abstract

Cells of bakers' yeast harvested from a chemically-defined medium were placed in buffer for sporulation experiments and in Wickerham's yeast nitrogen base for growth experiments. The metabolites were included in these two solutions in concentrations up to 1% usually, and growth and sporulation were compared under conditions as nearly equivalent as possible. Optimum sporulation was observed in 0.033–0.1% glucose, 1% pyruvate, 0.033% ethanol, 0.033% acetaldehyde, and 0.33% acetate. Optimum growth in one day occurred in 1% glucose, 1% pyruvate, 0.1–0.33% ethanol, 0.0033% acetaldehyde, and 0.033% acetate. Very few asci were found in 0.33% glucose or in 1% ethanol, but cells transferred to buffer after one day in such solutions sporulated well. The addition of 0.33% glucose or 1% ethanol to 0.3% acetate suppressed sporulation. In 0.1% acetaldehyde no sporulation or growth was evident, with or without the addition of 0.3% acetate. Acetoin, 2,3-butylene glycol, and ethylene glycol had no marked effect on sporulation and growth. Sporulation and growth were inhibited by 7×10^{-4} *M* fluoroacetate with 0.3% acetate or 0.3% ethanol as the carbon source. Inhibition was much greater when the acetate concentration was decreased to 0.03%. Sporulation in glucose was inhibited by fluoroacetate, while growth in glucose was little affected. With acetate as the carbon source, sporulation was more sensitive than growth to urethane, while the reverse was found with azide, malachite green, cyanide, and dinitrophenol. The two processes seemed about equally sensitive to arsenite, *p*-nitrophenol, and malonic acid. Most of the asci that formed in glucose solutions were two-spored. In acetate, ethanol, and pyruvate three- and four-spored asci usually predominated, except in the weaker concentrations.

Introduction

It was shown by Stantial (36) that some strains of yeast will sporulate readily in dilute solutions of certain sugars, or acetates, or both together, and this has been abundantly confirmed (8, 1, 32, 11, 37, 24, 2). That is, a supply of assimilable nitrogen and of minerals, other than that contained in the cells, is not required. Several other indications of basic differences between the metabolism of sporulation and that of growth in *Saccharomyces cerevisiae* have been pointed out by Miller *et al.* (29). (*a*) Optimum sporulation occurs in a hexose concentration that is much below the optimum for growth. One part in 2000 glucose is about optimum for sporulation, but at one part in 200

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few asci form. (*b*) Ethanol in a concentration of 1% suppresses sporulation, although this compound can serve as a carbon source for aerobic growth. (*c*) Abundant growth occurs in a nutrient medium with 0.5% ammonium sulphate as the nitrogen source, but 1/50th of this amount decreases sporulation when added to a sporulation medium.

Sporulation in *Saccharomyces cerevisiae* has been shown by Winge (39) to involve the formation of haploid spores from diploid vegetative cells, and is the equivalent of gametogenesis in higher organisms. Yeast provides an excellent experimental subject for the study of the metabolic factors that determine whether a diploid cell will divide vegetatively or undergo gametogenesis, for several reasons: it is unicellular and therefore more directly sensitive to environmental influences than are multicellular organisms; it can be cultivated under a wide variety of conditions; and its metabolism has been so intensively studied that the major features may be regarded as established. The purpose of the present study was to attempt a physiological comparison of sporulating and growing yeast. The experimental approach adopted was to employ certain compounds that are known to be assimilated as carbon sources by intact yeast cells and compare the growth and sporulation responses over a wide range of concentrations. This method has been accompanied by experiments in which inhibitors having more or less specific effects on yeast metabolism were included in sporulation and growth media.

Materials and Methods

The yeast culture employed, designated as F49, was isolated from Fleischmann packaged yeast. Storage cultures were maintained in the refrigerator on butts of nutrient medium covered with mineral oil as recommended by Henry (17). Stocks in current use were grown on slants of Wickerham's (38) yeast nitrogen base (Difco) plus 1% glucose and 2% agar, and transferred every two or three days. Vegetative cells for inoculating growth or sporulation cultures were harvested from two-day cultures in 6-oz. pharmaceutical bottles containing 10 ml. of yeast nitrogen base plus 1% glucose. Yeast nitrogen base, dissolved in *M*/30 phosphate buffer of pH 6 (unless otherwise stated) was used for growth experiments as the basal medium to which various carbon sources and other compounds were added. It is a chemically-defined, carbohydrate-free medium recommended for use in yeast taxonomy, containing salts, trace elements, vitamins, and amino acids with 0.5% ammonium sulphate as the nitrogen source. Although the manufacturers recommend filter-sterilization, observation showed that our yeast grew quite as well on this medium when it was autoclaved for 10 min. at 15 lb. pressure, so this more convenient sterilization method was used in the later experiments.

The growth cultures, 10 ml. in volume, were prepared in 6-oz. pharmaceutical bottles. Approximately 24 hr. after a growth experiment was set up, the numbers of cells per milliliter were estimated with a hemacytometer counting chamber. Buds were not counted unless they were at least half as large as

the mother cells. The values for growth given in the tables and figures were obtained by deducting the 100,000 cells per milliliter added as inoculum from the cell population density present one day later.

Sporulation cultures were prepared in $M/30$ phosphate buffer of pH 6 unless otherwise stated, and dispensed in 8-ml. volumes in 6-oz. pharmaceutical bottles except for a few experiments in which shell vials (14 × 45 mm.) containing 0.8 ml. of liquid were used. They were inoculated with approximately 4,000,000 cells per milliliter since this cell population density has been found to be optimum for sporulation in acetate solutions (29). Sporulation was estimated by examining microscopically 200 cells from each vial or bottle, usually five to seven days after the experiment was set up. Records were made of the frequency of vegetative cells, one-spored asci, two-spored asci, and three- or four-spored asci. As pointed out by Kleyn (24) it is difficult in practice to distinguish with certainty between three- and four-spored asci. The frequency of one-spored asci was always low, rarely exceeding 1–2%.

Inhibitors to be included in growth and sporulation cultures were added about 20 min. before the carbon source (usually acetate), since the effectiveness of some inhibitors, for instance fluoroacetate (22), is less if they are added after the substrate.

When volatile substances such as ethanol, acetaldehyde, cyanide, etc., were added to the cultures, screw caps were placed tightly on all the bottles. Experiments showed that neither sporulation nor one-day growth were noticeably affected by thus preventing the exchange of air. Since the total volume of a bottle was 185 ml., sufficient oxygen was presumably present for cultures 8 or 10 ml. in volume. Although the screw caps would prevent loss from the bottles of volatile substances, the concentrations of these included in the cultures must have been slightly lessened by evaporation into the air in the bottles.

All cultures were incubated at 27 ± 1° C., and the bottles were placed on their flat sides to ensure aeration of the liquid. The experiments were done in duplicate.

Results

Sporulation and Growth in Glucose and Acetate

In Table I are shown the results of experiments comparing sporulation and growth in varying concentrations of glucose and acetate. It is evident that glucose was a much better carbon source for growth than acetate at the two highest concentrations tested (0.33% and 1.0%) but that little difference was apparent at the lowest concentration. Sporulation in glucose showed a marked decline at the higher concentrations, which was not found with acetate. Few of the asci formed in glucose were three- and four-spored while in acetate such asci comprised about half of the total except at the lower concentrations. When cells were separated from the glucose solutions after one day by centrifuging, washed, and resuspended in buffer, the cells that had been exposed to the higher concentrations sporulated abundantly. The sporulation

TABLE I

INFLUENCE OF TWO CARBON SOURCES, GLUCOSE AND ACETATE, ON SPORULATION AND GROWTH

Amount of glucose or acetate included in sporulation or growth medium	Sporulation after 6–7* days in carbon source		Sporulation after one day in carbon source and 5–6† days in buffer		Growth, millions of cells per ml.
	Total asci, %	3- and 4-spored asci, %	Total asci, %	3- and 4-spored asci, %	
(A) None (control)	1.3	0.3			0.3
0.01% glucose	26.8	0.5	20.0	0	2.6
0.033% "	38.0	0.5	22.3	2.0	8.2
0.1% "	38.0	3.5	21.5	0.5	12.1
0.33% "	5.0	0.8	24.3	0.3	20.9
1.0% "	1.0	0.3	39.3	0.5	50.9
(B) None (control)	3.0	0.3			0.06
0.01% acetate	18.0	5.3	13.0	4.0	2.2
0.033% "	32.0	12.5	22.8	9.3	4.0
0.1% "	42.3	18.8	26.5	10.3	3.6
0.33% "	56.0	32.5	21.8	11.5	3.6
1.0% "	45.0	22.0	45.0	19.0	2.4
(C) 0.3% acetate (control)	19.8	14.7			
0.3% acetate + 0.0033% glucose	12.3	6.8			
0.3% acetate + 0.01% glucose	11.3	4.8			
0.3% acetate + 0.033% glucose	16.0	5.3			
0.3% acetate + 0.1% glucose	13.6	1.3			
0.3% acetate + 0.3% glucose	0.3	0			

**In A, sporulation was determined after six days, and in B and C, after seven days.*
†In A, sporulation was determined after five days in buffer, and in B, after six days in buffer.

of cells at concentrations below 0.33% was somewhat lessened by this treatment. When cells were removed from acetate solutions in this manner, no increase resulted with any initial concentration, and there seemed, in fact, to be a consistent decrease at all concentrations below 1%. The treatment did not affect the proportion of three- and four-spored asci in glucose or in acetate.

The effect on sporulation of adding a series of glucose concentrations to 0.3% acetate is shown at the bottom of Table I. The yield of asci was not strongly diminished unless the glucose concentration was greater than 0.1%. In 0.3% acetate most of the asci contained three or four spores but the inclusion of glucose reduced the frequency of such asci. The foregoing results indicate that glucose in moderate concentrations (0.33, 1.0%) inhibits sporulation, whereas it stimulates at 0.01 to 0.1%. Since the inclusion of glucose in acetate solutions decreases the abundance of three- and four-spored asci, this may likewise be an inhibition.

TABLE II

SPORULATION OF CELLS AFTER VARIED TREATMENTS WITH SODIUM ACETATE* AND GLUCOSE*

Treatment of cells	Total asci, %	3- and 4-spored asci, %
Buffer for 7 days	0	0
Acetate for 7 days	31.3	17.8
Acetate for 1 day; buffer for 6 days	27.8	16.3
Glucose for 7 days	1.8	0.8
Glucose for 1 day; buffer for 6 days	6.8	1.8
Acetate + glucose for 7 days	0.3	0.3
Acetate + glucose for 1 day; buffer for 6 days	28.8	0.8
Buffer for 6 days	0.8	0
Acetate for 1 day; buffer for 5 days	28.3	19.8
Glucose for 1 day; buffer for 5 days	6.5	0
Acetate + glucose for 1 day; buffer for 5 days	23.8	5.0
Acetate for 1 day; 0.1% glucose for 5 days	20.3	14.0
Glucose for 1 day; acetate for 5 days	23.5	6.3

**Acetate and glucose were used in 0.3% concentration in both experiments except where otherwise stated.*

Glucose inhibition of sporulation was investigated further in two experiments summarized in Table II. The removal of an inhibiting concentration (0.3%) of glucose after one day did not give as high yields of asci as in the foregoing experiments. When cells were removed from 0.3% glucose + 0.3% acetate after one day, the interference with three- and four-spored ascus development was still present, and it was not overcome when cells kept for one day in 0.3% glucose were placed in 0.3% acetate. On the other hand, when cells were removed from 0.3% acetate after one day and placed in 0.1% glucose there was no definite reduction in the frequency of three- and four-spored asci. After one day in 0.3% acetate, ascospores are usually just beginning to appear and by that time it may have been determined whether a given ascus would contain two spores or more.

Two substances known to affect glycolytic reactions were compared regarding their effects on growth and sporulation in glucose and acetate. The results are given in Table III. Sodium fluoride was somewhat more inhibiting to growth in acetate than in glucose. It did not overcome the inhibition of sporulation by glucose. Sporulation in acetate was less sensitive to fluoride than was growth. At 10^{-1} *M*, the strongest concentration used, asci were only about 18% as abundant as in the control, although the proportion of three- and four-spored asci seemed increased. Sodium iodoacetate gave results similar to fluoride. Growth in acetate was more sensitive to it than growth in glucose or sporulation in acetate. Glucose inhibition was not

TABLE III

EFFECT OF TWO INHIBITORS OF GLYCOLYTIC ENZYMES ON SPORULATION AND GROWTH IN ACETATE AND GLUCOSE

Inhibitor	0.3% sodium acetate			0.3% glucose		
	Sporulation		Growth, millions of cells per ml.	Sporulation		Growth, millions of cells per ml.
	Total asci, %	3- and 4-spored asci, %		Total asci, %	3- and 4-spored asci, %	
0	50.8	27.0	3.1	0.3	0	11.2
10^{-2} *M* fluoride	45.8	26.0	1.2	0	0	12.3
3.3×10^{-2} *M* fluoride	36.3	21.8	1.1	0	0	8.9
10^{-1} *M* fluoride	9.3	8.0	0.1	0	0	0
0	39.0	23.3	2.9	1.0	0.8	23.9
10^{-5} *M* iodoacetate	34.3	24.3	3.4			
3.3×10^{-5} *M* iodoacetate	33.3	21.8	1.6			
10^{-4} *M* iodoacetate	23.8	17.3	1.3	0.3	0	15.9
3.3×10^{-4} *M* iodoacetate	16.3	13.0	0.2	0	0	9.9
10^{-3} *M* iodoacetate	0	0	0.02	0	0	2.1

reversed by iodoacetate, and at the highest iodoacetate concentration (3.3×10^{-4} *M*) that did not give complete inhibition of sporulation in acetate, the proportion of three- and four-spored asci appeared increased.

Sporulation and Growth in Ethanol and Acetaldehyde

Certain yeasts are able to assimilate these substances under aerobic conditions, but except for the report by Stantial (36) that 10% "bad ascs" developed in 0.01% ethanol, they have not hitherto been found to stimulate sporulation. Stantial observed inhibition of sporulation by 1% ethanol and this has been

TABLE IV

SPORULATION AND GROWTH IN ETHANOL AND ACETALDEHYDE

Concentration in sporulation or growth medium, %	Ethanol			Acetaldehyde		
	Sporulation		Growth, millions of cells per ml.	Sporulation		Growth, millions of cells per ml.
	Total asci, %	3- and 4-spored asci, %		Total asci, %	3- and 4-spored asci, %	
0	0	0	0.1	0.8	0	0
Sodium acetate (Control)*	36.8	27.3	2.8	39.8	27.5	2.9
0.001				1.8	0	0.57
0.0033	3.5	0.5	1.1	1.5	0.3	0.83
0.01	28.5	7.5	1.9	14.5	5.8	0.48
0.033	43.5	26.3	1.9	19.3	9.8	0.29
0.1	22.0	13.5	2.3	0	0	0
0.33	11.5	7.5	2.3			
1.0	1.0	0.5	1.4			

**For comparison, controls containing 0.3% sodium acetate were included in the ethanol experiment, and 0.1% in the acetaldehyde experiment.*

confirmed by others (2, 29). No stimulation by acetaldehyde was observed by Miller *et al.* (29) using slants of water agar plugged with cotton, but this may have been due to rapid loss of the compound through evaporation.

The results of experiments comparing sporulation and growth in varying concentrations of ethanol and acetaldehyde are shown in Table IV. Both compounds supported growth and sporulation at certain concentrations. With ethanol, abundant sporulation occurred from 0.01 to 0.1% and in this respect it resembled glucose (Table I). But it differed in allowing more sporulation at 0.33%, in not suppressing the formation of three- and four-spored asci, and in supporting more growth at 0.33% than at 1%. Acetaldehyde stimulated sporulation at concentrations of 0.01 and 0.033%. No asci were observed at 0.1%, presumably owing to the toxic effect of acetaldehyde (12) since some of the cells appeared disorganized. This would also explain why no growth occurred at that concentration. In fact, the growth optimum with acetaldehyde was much lower (0.0033%) than with any of the other carbon sources studied.

From these results one could entertain the hypothesis that inhibition of sporulation by glucose is due to the formation of acetaldehyde, particularly since Pickett and Clifton (33) found evidence for the formation of acetaldehyde by yeast cells oxidizing glucose. But the inclusion of substances reported to inhibit carboxylase (salicylate, 1-naphthol-2-sulphonic acid, quinone) in sporulation cultures containing 0.3% glucose did not cause sporulation to proceed, nor did an acetaldehyde fixative (semicarbazide hydrochloride).

The possibility that ethanol inhibition of sporulation results from its conversion to acetaldehyde by alcohol dehydrogenase was investigated by adding semicarbazide and iodoacetate to sporulation cultures containing 0.3%

TABLE V

EFFECT OF IODOACETATE AND SEMICARBAZIDE ON SPORULATION AND GROWTH IN 0.3% ETHANOL

Concentration added to sporulation or growth medium, *M*	Iodoacetate			Semicarbazide		
	Sporulation			Sporulation		
	Total asci, %	3- and 4-spored asci, %	Growth*	Total asci, %	3- and 4-spored asci, %	Growth*
0	24.0	12.3	0.84	20.8	13.0	1.3
10^{-5}	11.0	6.3	0.38			
3.3×10^{-5}	6.3	3.5	0.10			
10^{-4}	0.8	0	0			
3.3×10^{-4}	0	0	0.02			
10^{-3}	0	0	0.05	13.8	7.3	0.12
3.3×10^{-3}				8.3	3.5	0.16
10^{-2}				0.5	0	0.09
3.3×10^{-2}				0.3	0	0.05
10^{-1}				0.3	0	0

**Increase in cell population density (millions per ml.) in one day.*

ethanol. Iodoacetate is known to be a strong inhibitor of alcohol dehydrogenase (21, 20). The results, shown in Table V, give no evidence of reversal of ethanol inhibition of sporulation by either compound, but sporulation (and also cell multiplication in growth cultures) were depressed at all concentrations. In comparison, sporulation and growth in acetate (Table III) were much less sensitive to iodoacetate. In Table VI, A, are shown the results obtained with another acetaldehyde-fixing agent, sodium sulphite, added to sporulation cultures containing 0.3% ethanol. Again, no increase in sporulation was noted.

In the foregoing experiments with ethanol the concentrations of iodoacetate, semicarbazide, and sulphite may have been so strong as to diminish the amount of acetaldehyde available in the cells from a value above to a value below the optimum for sporulation so that no stimulation could have resulted. To investigate this possibility, a further experiment was done in which 0.3% acetate was included in sporulation cultures containing 1% ethanol and sufficient iodoacetate or semicarbazide to cause partial inhibition of sporulation in acetate and complete inhibition of sporulation in 0.3% ethanol. The results,

TABLE VI

SPORULATION IN VARIOUS COMBINATIONS OF ACETATE, ETHANOL, ACETALDEHYDE, IODOACETATE, SEMICARBAZIDE, AND SULPHITE

Treatment of cells	Sporulation	
	Total asci, %	3- and 4-spored asci, %
(A) Buffer only	0	0
0.3% ethanol	18.0	5.3
0.3% ethanol + 10^{-2} *M* sodium sulphite	5.8	2.5
0.3% ethanol + 10^{-1} *M* sodium sulphite	1.8	1.0
(B) Buffer only	0.3	0
0.3% acetate	22.3	17.3
1.0% ethanol	2.0	0.5
0.3% acetate + 1.0% ethanol	1.0	0.5
0.3% acetate + 10^{-4} *M* iodoacetate	14.8	12.3
0.3% acetate + 1.0% ethanol + 10^{-4} *M* iodoacetate	0.5	0.3
0.3% acetate + 10^{-2} *M* semicarbazide	6.1	2.2
0.3% acetate + 1.0% ethanol + 10^{-2} *M* semicarbazide	0.5	0
0.1% acetaldehyde	1.0	0.5
0.1% acetaldehyde + 10^{-4} *M* iodoacetate	0	0
0.3% acetate + 0.1% acetaldehyde	0.5	0.3
0.3% acetate + 0.1% acetaldehyde + 10^{-4} *M* iodoacetate	0	0
(C) Buffer only	0	0
0.3% acetate	26.5	20.3
0.3% ethanol	16.8	7.0
0.3% acetate + 0.3% ethanol	16.0	9.0
0.3% acetate + 0.3% ethanol + 10^{-4} *M* iodoacetate	7.0	4.0
0.3% acetate + 0.3% ethanol + 10^{-2} *M* sodium sulphite	13.5	9.8 0

shown in Table VI, B, indicate that neither iodoacetate nor semicarbazide counteracted the suppression of sporulation caused by ethanol. Table VI, C, gives the results of a similar experiment using 0.3% alcohol (a partially-inhibiting concentration), 0.3% acetate, 10^{-4} *M* iodoacetate, and 10^{-2} *M* sulphite. Here again there is no evidence that treatments which should hinder the formation of acetaldehyde from ethanol or restrict its accumulation in the cells can overcome ethanol inhibition of sporulation.

These results are in agreement with the view that inhibition of sporulation by ethanol results not from acetaldehyde formation but from some other reaction or reactions in which ethanol participates. On the other hand, the results indicate that in its stimulation of sporulation ethanol goes through the acetaldehyde stage. Martius and Lynen (28) found ethanol to be oxidized via acetate. Others (4, 35) have described enzymes in yeast capable of oxidizing acetaldehyde to acetate. One might expect, then, that in stimulating sporulation ethanol would go through the acetate stage, and this receives some support from experiments with fluoroacetate which inhibited sporulation (and also growth) in ethanol.

Since acetaldehyde prevents sporulation at much lower concentrations than does ethanol, it would hardly be expected to do so through forming ethanol, and this is in conformity with the observation (Table VI, B), that 10^{-4} *M* iodoacetate did not overcome acetaldehyde inhibition of sporulation. Further evidence that these two inhibitions are independent is supplied by the observation that acetaldehyde strongly inhibits growth at the same concentration that suppresses sporulation, while ethanol does not. In addition it has been found that inhibition by 0.3% ethanol can be overcome by removing the cells from it to buffer after one day. It resembles glucose in this respect. But with acetaldehyde, transfer of cells from a 0.1% concentration to buffer after one day did not cause sporulation to occur.

Butylene Glycol, Ethylene Glycol, Benzaldehyde, and Acetoin

In Table VII are shown the results of experiments done to determine the effect on sporulation and growth of 2,3-butylene glycol, ethylene glycol, benzaldehyde, and for comparison, acetaldehyde. The first two compounds

TABLE VII

SPORULATION AND GROWTH IN 0.3% ACETATE PLUS 2,3-BUTYLENE GLYCOL, ETHYLENE GLYCOL, BENZALDEHYDE, AND ACETALDEHYDE

Concentration of compound added to 0.3% acetate solution, %	Butylene glycol		Ethylene glycol		Benzaldehyde		Acetaldehyde	
	Sporulation, %	Growth*	Sporulation, %	Growth*	Sporulation, %	Growth*	Sporulation, %	Growth*
0 (without acetate)	0	0.07	0	0.12	0	0.04	0.5	0.07
0	31.0	3.9	19.8	3.7	36.8	2.7	30.0	2.8
0.01	34.5	2.8	22.5	3.1	42.3	2.4	32.3	2.1
0.033	21.8	3.1	19.8	2.7	34.5	0.18	27.0	0.92
0.1	30.0	3.1	11.8	3.0	0	0	1.5	0

**Increase in cell population density (millions per ml.) in one day.*

had little or no effect on either process. Both benzaldehyde and acetaldehyde were strongly inhibiting to sporulation and growth at 0.1%, but not at 0.01%. Genevois (12) states that acetaldehyde is toxic to yeast at concentrations above 10^{-2} *M* (= 0.044%). Neither compound inhibited sporulation at the 0.033% concentration, while growth was strongly inhibited, especially by benzaldehyde. It is of interest to compare these results with those in Table IV showing sporulation and growth at the same acetaldehyde concentrations without added acetate. With or without acetate, less growth was observed in 0.033% acetaldehyde than in 0.01%. Sporulation was somewhat greater at the former concentration in the absence of acetate, but not in its presence. Acetaldehyde was less effective as an inhibitor of growth than benzaldehyde, and the difference would be accentuated by expressing the concentrations on a molar basis. However, respiration and assimilation of some of the acetaldehyde would possibly reduce its concentration inside the cells, making it seem less toxic.

In another experiment the compound acetoin was found to cause a moderate suppression of sporulation when included in 0.3% concentration in acetate sporulation cultures, but it did not affect growth in growth cultures.

Sodium Pyruvate and Sodium dl-Lactate

In Table VIII five concentrations of pyruvate and one of lactate are compared with acetate (0.1 and 0.3%) in respect of their stimulation of sporulation and growth. Pyruvate supported both processes, particularly at the highest concentration used (1%). Comparing the results at the 0.1% concentration of pyruvate and acetate, it is seen that while growth was similar in both, sporulation was much less abundant in pyruvate. An inhibition probably is not involved since more asci formed in the higher pyruvate concentrations. Barron *et al.* (3) found pyruvate to be consumed much more rapidly by yeast cells at pH 4 than at pH 6 and attributed it to increased

TABLE VIII

SPORULATION AND GROWTH IN SODIUM PYRUVATE AND SODIUM LACTATE

Compound added to sporulation or growth medium	Sporulation		Growth, millions of cells per ml.
	Total asci, %	3- and 4-spored asci, %	
Control	0.3	0	0
0.1% acetate	35.5	29.5	2.8
0.3% acetate	26.8	17.5	2.2
0.01% pyruvate	0.5	0.3	0.5
0.033% pyruvate	3.8	1.5	1.1
0.1% pyruvate	10.0	3.0	2.8
0.33% pyruvate	19.0	12.5	3.5
1.0% pyruvate	27.0	20.5	4.9
0.1% lactate	20.8	6.8	1.3

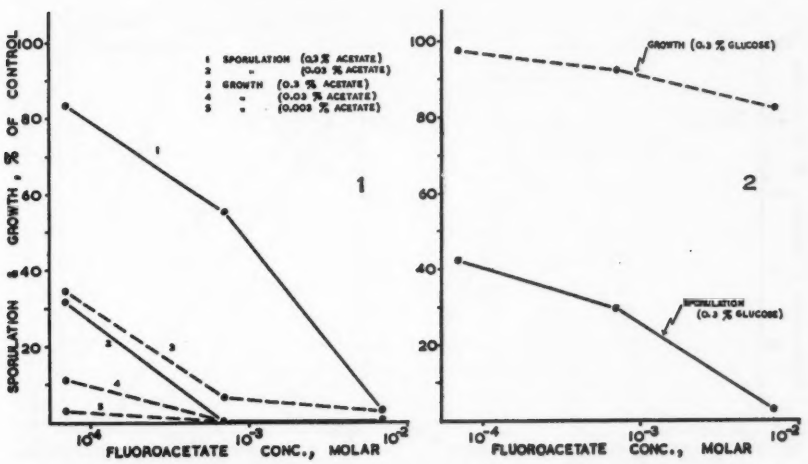
permeability in the more acid environment. Since the sporulation and growth media used here were prepared with $M/30$ phosphate buffer at pH 6, the permeability factor should have been uniform unless affected by the differences in composition of the two media.

Growth in lactate was difficult to count accurately since the buds tended not to separate from the mother cells, forming small clumps. Sporulation was rather less abundant than in the same concentration (0.1%) of acetate, but it was more abundant than in 0.1% pyruvate.

Sodium Fluoroacetate

To obtain comparative results with 0.3% acetate and 0.3% glucose, advantage was taken, in the experiments with fluoroacetate, of the fact that cells removed from acetate and glucose after one day will sporulate. A concentration of 0.3% glucose prevents sporulation if the cells are left in it. One day after an experiment was set up, the sporulation cultures containing acetate or glucose with or without fluoroacetate were centrifuged to remove the cells, which were then placed in buffer.

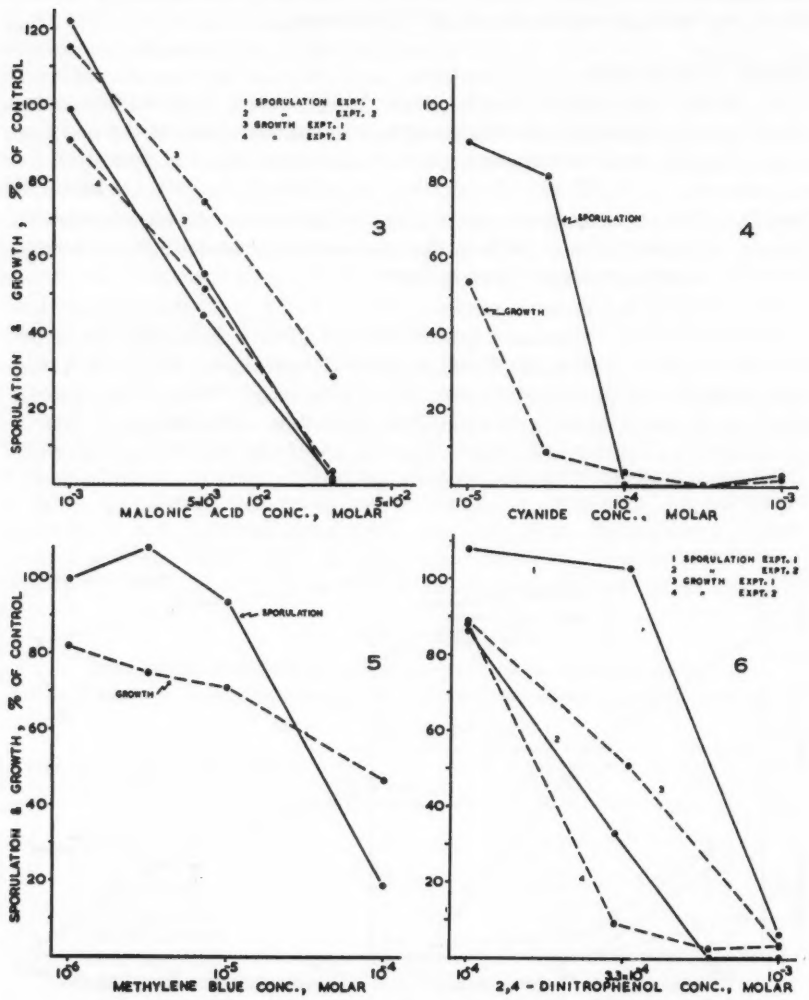
The results with acetate as nutrient, given in Fig. 1, show that fluoroacetate in low concentration depressed sporulation and growth, especially the latter. At a very low concentration of acetate (0.03%) sporulation and growth were more sensitive to fluoroacetate than at 0.3% acetate. When 0.3% glucose served as nutrient, sporulation seemed more sensitive to fluoroacetate than at the same concentration of acetate. Growth in glucose was not very sensitive to the concentrations of fluoroacetate employed.



FIGS. 1 and 2. Sporulation and growth in acetate (FIG. 1) and 0.3% glucose (FIG. 2) at varying concentrations of fluoroacetate. (In all figures, solid lines indicate sporulation, and broken lines indicate growth.)

Malonic Acid

The experiments with this compound were done in phthalate buffer at pH 4 since it penetrates yeast cells very slowly in less acid environments. Sporulation and growth were both inhibited (Fig. 3). In the results of one experiment sporulation appeared somewhat more sensitive than growth, but in the other both were similar.

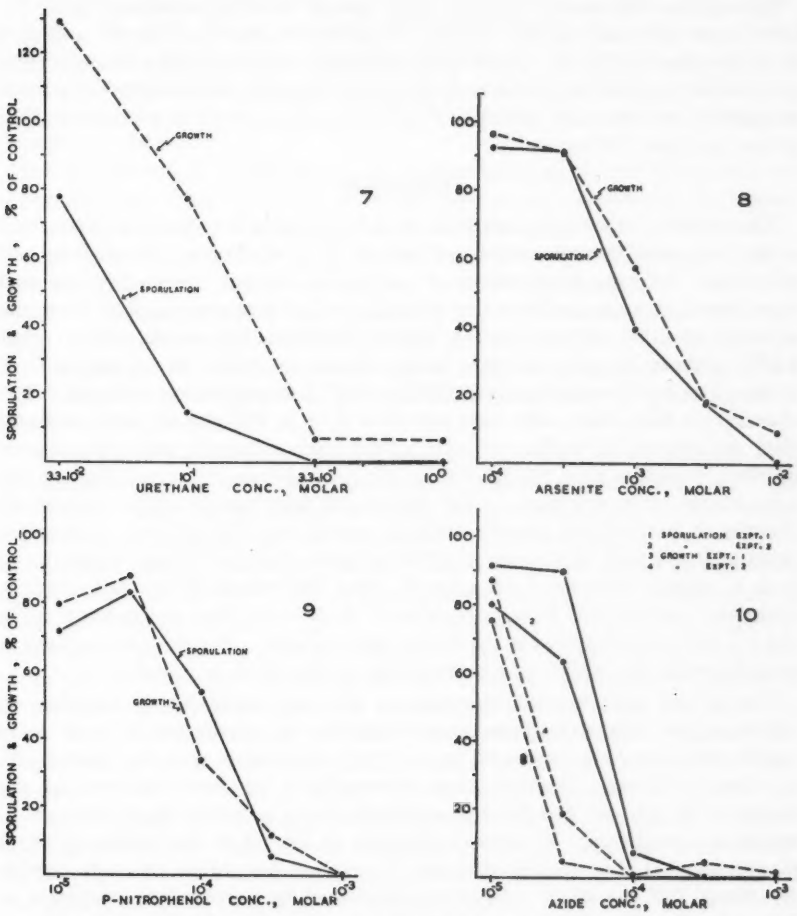


FIGS. 3–6. Sporulation and growth in 0.3% acetate at varying concentrations of malonic acid, cyanide, methylene blue, and 2,4-dinitrophenol.

Potassium Cyanide and Methylene Blue

The results of an experiment comparing growth and sporulation in several concentrations of cyanide (Fig. 4) show that both processes were strongly inhibited at 10^{-4} *M*, and that 10^{-5} *M* partially inhibited growth but had little effect on sporulation.

Fig. 5 shows the effect of methylene blue on sporulation and growth. Both were inhibited at 10^{-4} *M*. Since Chen and Tang (6) found that this redox dye overcame cyanide inhibition of oxygen uptake by yeast respiring acetate, an attempt was made to reverse cyanide inhibition of sporulation by adding to



FIGS. 7–10. Sporulation and growth in 0.3% acetate at varying concentrations of ethyl urethane, arsenite, *p*-nitrophenol, and azide.

the cultures containing 10^{-4} *M* cyanide an amount of the dye (10^{-5} *M*) which, from Fig. 5, would not in itself be inhibiting. This treatment did not, however, overcome the cyanide inhibition.

2,4-Dinitrophenol

The results of two experiments comparing the effect of this substance on growth and sporulation (Fig. 6) show that both were almost completely prevented by 10^{-3} *M*. Sporulation was, on the average, less sensitive than growth.

Other Inhibitors

Sporulation was more sensitive than growth to ethyl urethane (Fig. 7), while both processes seemed equally sensitive to arsenite (Fig. 8) and also to *p*-nitrophenol (Fig. 9). Azide (Fig. 10) inhibited growth more strongly than sporulation, and the same was true of malachite green, which inhibited growth completely and reduced sporulation to 71% of the control at a concentration of one part per million.

Discussion

The experimental work described above has mainly concerned a comparison of the responses of sporulation and growth to a variety of metabolites and inhibitors. Glucose gave results of particular interest, since very few asci were found in a concentration of 0.33%, and no increase resulted from the addition of 0.3% acetate to the glucose solution. It seems evident that 0.33% glucose in some manner brings about an inhibition of sporulation, although at lower concentrations (0.01 to 0.1%) it is quite stimulating. The observation that when cells were placed in 0.33 or 1% glucose for a day and then transferred to buffer without added carbon source good sporulation resulted, indicates that the inhibiting effect of glucose is exerted against the later stages of sporulation. Cells sporulated very poorly when transferred directly to buffer from growth medium containing 1% glucose. A possible explanation is that in glucose metabolism some substance may be produced that is rapidly consumed for growth when the necessary nitrogen source, vitamins, and mineral salts are present. But when they are lacking, as in the 1% glucose solution, the substance accumulates. It may then be used in sporulation if the inhibiting concentration of glucose is removed.

Foster (10) has proposed the concept of shunt metabolism, according to which excess substrate causes some metabolic intermediates to form more rapidly than they are consumed so that they accumulate and may participate in other reactions. Perhaps some intermediate (or intermediates) in the pathway of glucose breakdown accumulates in amounts large enough to suppress sporulation. It seemed plausible at first that the inhibiting effect of glucose was due to acetaldehyde or ethanol formation through aerobic glycolysis, but the effect was not counteracted by carboxylase inhibitors or by semicarbazide. Moreover, although acetaldehyde and ethanol should also form from pyruvate, the latter substance did not inhibit sporulation. Two

other compounds, acetoin and 2,3-butylene glycol, known to be formed during yeast fermentation, had little or no effect on sporulation. Two inhibitors of glycolysis, iodoacetate and fluoride, failed to reverse the suppression of sporulation by glucose, but this would be expected if the postulated substance was formed at a stage between glucose and the reactions sensitive to iodoacetate and fluoride in the Embden–Meyerhof pathway. Other possible explanations are that glucose interferes with sporulation through another metabolic pathway, or through some influence of the glucose molecule itself. The inhibiting concentration of glucose (0.3%) is so low that an osmotic effect seems unlikely.

Ethanol resembled glucose in suppressing sporulation at moderate concentrations. As with glucose, removing the cells to buffer after a day in ethanol overcame the effect, while treatments that should hinder acetaldehyde formation from ethanol did not overcome it. Thus it seems more probable that some other process in which ethanol participates hinders sporulation, though not growth.

In view of the great effectiveness of acetate as a sporulation stimulant, it is of interest that it supports growth rather poorly as compared with glucose. Cell multiplication in one day in a medium containing 0.3% glucose is from 5 to 10 times as great as in 0.3% acetate (or pyruvate). Gilvarg and Bloch (13) concluded that while the amino acids phenylalanine, tyrosine, and glycine were readily formed from glucose by yeast, when acetate served as the carbon source they were synthesized with difficulty. This may explain the slow rate of growth observed in the present study with acetate, ethanol, and acetaldehyde. With pyruvate, low permeability of the cell walls at pH 6 could also be involved.

The proportion of three- and four-spored asci was much less when cells were sporulated in glucose than with any of the other compounds studied. The addition of acetate to the glucose solutions did not overcome the effect, which indicates that it is not due to the need for some metabolite more readily formed from acetate than from glucose. Transfer of cells to acetate after one day in an inhibiting concentration of glucose did not result in a high yield of three- and four-spored asci. That is, the proportion of these was characteristic of glucose rather than of acetate. This is evidence that early stages in spore formation determining the number per ascus are not inhibited by glucose as are the later stages that result in the development of visible spores. The latter can, however, proceed if the glucose is removed. Fluoride and iodoacetate at certain concentrations increased the proportion of three- and four-spored asci formed in acetate. These observations may be valuable for a future biochemical clarification of the processes that determine the number of spores per ascus. It is of interest that Kleyn (24) obtained fewer three- and four-spored asci when glucose was added in several concentrations to a medium that allowed both growth and sporulation.

Evidence has been obtained in recent years which indicates that fluoroacetate prevents the functioning of the tricarboxylic acid cycle by

forming, through combination with oxaloacetic acid, a fluorocitric acid that competitively inhibits aconitase (31). Thus its inhibition of growth and sporulation in acetate solutions gives some indication that the cycle is concerned in both processes under the conditions of the experiments. The finding that at a low concentration of acetate (0.03%) sporulation and growth were more sensitive to fluoroacetate than at 0.3% is in conformity with the conclusion of Kalnitsky and Barron (22) that inhibition by fluoroacetate of acetate respiration in yeast is competitive. When glucose served as nutrient, fluoroacetate restricted growth less than it did sporulation. This would suggest that while glucose can support growth without being oxidized via the tricarboxylic acid cycle, the latter pathway must be followed for sporulation to result. Kalnitsky and Barron (22) observed that oxidation of glucose by yeast was not as strongly inhibited by fluoroacetate as the oxidation of acetate and that ethanol resembled glucose in this respect. Similarly, Black and Hutchens (5) found that ethanol oxidation was not strongly inhibited. In the present study, however, growth in 0.3% ethanol was reduced to 4.8% of the control and sporulation to 23.4% of the control by 7×10^{-4} *M* fluoroacetate. Ethanol thus resembled acetate rather than glucose in its response to fluoroacetate.

Malonic acid, a well-known competitive inhibitor of succinic dehydrogenase, was quite effective in inhibiting sporulation and growth in acetate, and this observation, taken in conjunction with the results obtained with fluoroacetate, is in accord with the hypothesis that the tricarboxylic acid cycle is involved in the stimulation of sporulation by acetate. The writers have, however, been unable to obtain sporulation or growth in media containing as carbon sources any of the following five tricarboxylic acid cycle intermediates: citric, α-ketoglutaric, succinic, fumaric, and malic acids. This may seem in conflict with the foregoing hypothesis, but it has been shown (7, 27, 28, 26) that intact yeast cells are almost impermeable to these acids.

Conflicting reports (25) have been given concerning the importance of the tricarboxylic acid cycle in yeast. Krebs *et al.* (26) were of the opinion that some of the reactions of the cycle in yeast were too slow to account for the observed rate of acetate consumption and concluded that it served mainly to furnish metabolic intermediates. Hansen and Nossal (15) presented evidence that the technique used by Krebs *et al.* disorganized the cells to such a degree that the enzymes of the cycle could not function normally. From their work the tricarboxylic acid cycle is no longer precluded from being the main pathway of acetate respiration in yeast.

The major portion of bakers' yeast respiration under normal conditions evidently passes through the cytochrome system. Haas (14) was able to account for the whole oxygen uptake in terms of the oxidation and reduction of cytochrome *c*, and Keilin (23) observed that the cytochrome oxidase activity of the cells was inhibited by cyanide in the same manner as the oxygen uptake. The inhibitions observed with dilute cyanide thus indicate a functioning of the cytochrome system during sporulation and growth in acetate. Of interest

here is the report of Ephrussi and Hottinguer (9) on certain slowly-growing yeast mutants that differed from the original strain in lacking a cyanide-sensitive respiration owing partly to a deficiency in cytochrome oxidase. They were unable to induce sporulation of these mutants.

The suppression of sporulation by dinitrophenol, which is known to uncouple oxidative phosphorylation from respiration (30), suggests that at least one of the functions of acetate in stimulating sporulation may be the generation of phosphate bond energy. Winzler (40) found that 5×10^{-4} *M* dinitrophenol inhibited the utilization of acetate for synthesis by yeast, although its oxidation was complete.

Ammonium salts were shown by Saito (34) to have a depressing effect on the sporulation of cells of *Saccharomyces mandshuricus* spread on moistened gypsum blocks, and Elder (8) later observed the same effect with cells sporulating in acetate–glucose solution. This was confirmed by Miller *et al.* (29). During the present study further experiments showed sporulation in 0.1% acetate solution to be inhibited almost completely by ammonium sulphate at concentrations as low as 0.03%. In a synthetic medium similar to the presporulation medium but containing 0.3% acetate as the carbon source, and no nitrogen source, the writers have obtained 38.5% sporulation, which indicates that components of the presporulation medium other than glucose and ammonium sulphate do not inhibit sporulation. Possibly ammonium sulphate interferes with sporulation by promoting the synthetic reactions of growth which could then compete with those of sporulation for metabolites necessary to both.

Two reasons are apparent for the failure to observe sporulation in growth media such as the presporulation medium used here, namely, the presence of fairly large amounts of sugar and available nitrogen. Evidently many other fungi resemble yeast in this respect since Hawker (16) has pointed out that, in general, fruiting of fungi is inhibited by carbohydrate at concentrations favoring vegetative growth, and Hirsch (18) stated that gamete differentiation in many fungi does not occur unless the nitrogen content of the medium is low or depleted.

Acknowledgments

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EFFECTS OF DEPTH OF STYLET PENETRATION ON APHID TRANSMISSION OF POTATO VIRUS Y[1]

By R. H. E. Bradley[2]

Abstract

The aphid *Myzus persicae* (Sulz.) most often became infective with potato virus Y on inserting the stylets *only superficially* into infected tobacco plants after a period without food; the further the stylets penetrated into the plant, the less likely the aphid was to be infective. When epidermis was removed from infected plants, aphids acquired virus Y as readily from the exposed mesophyll as from the piece of removed epidermis. *M. persicae* can feed by inserting its stylets through a membrane and into tobacco plants. But aphids rarely became infective with virus Y when they inserted their stylets through a membrane and into infected plants; and infective aphids rarely transmitted the virus when they inserted their stylets through a membrane into susceptible plants. Yet infective aphids that penetrated a membrane could afterwards infect a plant not covered with a membrane. Also, aphids did not become infective when they penetrated the deeper tissues of infected plants that had had the virus in the superficial tissues inactivated by ultraviolet radiations. The main conclusion is that *M. persicae* rarely becomes infective with virus Y or transmits it after the stylets penetrate beyond the first layer of plant cells.

Introduction

Many aphid-borne viruses are readily acquired and transmitted when the vectors make brief attempts to feed. Feeding attempts of less than half a minute are adequate, the stylets usually penetrating only part way through the epidermal layer (4). Clearly then aphids acquire such viruses from the epidermis, and infections occur when virus passes from the aphid to the epidermis of susceptible plants. There is no direct evidence that aphids transmit such viruses *from* or *to* subepidermal tissues, and it has been suggested that they may not do so (6). Described herein is a study of this problem using green peach aphids transmitting potato virus Y.

Materials and General Methods

The strain of potato virus Y as well as the general methods and precautions against error have been described (6). All transmissions of virus Y were *from* and *to* White Burley tobacco plants (*Nicotiana tabacum* L.). Wingless adult aphids or late instar nymphs were used from a culture of *Myzus persicae* (Sulz.) maintained on turnips. All aphids were kept without food for one to several hours before the experiment, as this increases their efficiency as vectors (13).

The distance an aphid's stylets were inserted was determined by anesthetizing the aphid *in situ* before it withdrew its stylets. An aphid to be so anesthetized was covered for 10 sec. with the open end of a rubber tube from which carbon dioxide was slowly passing. This immobilized the aphid at once. While

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Contribution No. 3428, Entomology Division, Science Service, Department of Agriculture, Ottawa, Canada.
[2]*Associate Entomologist, Field Crop Insect Section, Entomology Laboratory, Fredericton, N.B.*

it remained anesthetized, it was removed with a brush and viewed under 100 × magnification to measure the distance the stylets extended beyond the end of the labium. This distance was measured to ± 5μ with an ocular micrometer. To establish that this distance was the same as that when the stylets were inserted before the aphid was anesthetized, single aphids were observed under a microscope as they inserted their stylets through a membrane into a liquid (4, 10); the movement of the stylets during penetration stopped immediately the aphid was surrounded with carbon dioxide, there being no withdrawal of the stylets. Moreover, the distance the stylets were inserted was the same as the distance they extended beyond the end of the labium after the aphid was removed. Anesthetized aphids recovered within a few minutes, after which they were tested for infectivity.

In experiments in which aphids inserted their stylets through a membrane into tobacco plants, a piece of membrane about 2 cm. wide was pressed into close contact with the moistened surface of a leaf. Aphids were confined on the membrane with a glass ring 1 cm. in diameter and 0.3 cm. long. One end of the ring was placed on the membrane and, after aphids were placed in the ring, the open end was covered with a glass cover slip. The membranes used were epidermis from tobacco and tulip plants, onion epidermis, and a synthetic material, parafilm (Fisher Scientific Co. Ltd., Montreal, Que.). The methods of obtaining epidermis free from other tissues have been described (4, 10). Parafilm is easily stretched to the desired thickness. After preparation all membranes were 10 to 20μ thick, this being less than that of the epidermis of the plants on which the membranes were placed.

Ultraviolet irradiation was done with a Model V-41 low-pressure mercury-vapor discharge lamp (Ultra-Violet Products Inc., South Pasadena, Calif.). The makers claim that 89.7% or more of the radiations from this lamp are of the 2537 A wave band region.

Uptake of Virus

Aphid-borne viruses have not been detected *within* their vectors by chemical or physical means. The only aphid-borne virus within an aphid that can be studied at present is that which the aphid can inoculate into susceptible plants and thereby cause infection. Hence, *uptake of virus* in this paper refers only to virus acquired in such a way that it can later be transmitted by the aphid.

Depth of Stylet Penetration

Table I shows that the highest percentage of infective aphids spent less than a minute on the infected plant, none inserting its stylets over 21μ. The likelihood of an aphid being infective decreased the deeper the stylets penetrated and as the time on the infected plant increased. Periods on infected plants were recorded to the nearest minute except those of less than a minute, which were recorded to the nearest five seconds. From 10 to 50 aphids were tested in this manner on some 20 occasions. The distances the stylets penetrated ranged from less than 5 to 370μ. Cross sections of the infected

leaves showed that the stylets would not have pierced the epidermis when they were inserted less than 21μ, and that they probably reached the mesophyll at 21–100μ, and the phloem at over 100μ.

Aphids were also anesthetized after several hours on infected plants to determine the depth of stylet penetration after which they were tested for infectivity. Most of these aphids had their stylets inserted over 25μ. The small percentage of aphids with their stylets inserted less than 25μ were about 10% infective. By comparison, there were only two infective aphids out of 400 that had their stylets inserted over 25μ. Aphids with their stylets inserted less than 25μ were penetrating through the epidermal layer. The longer aphids are on a plant the more of them have their stylets inserted into subepidermal tissues (Table I). This is to be expected, for once an aphid selects a feeding site it usually inserts its stylets to the phloem from which *M. persicae* is believed to imbibe its food. Regardless of how long aphids are on a plant, however, usually some will be changing their feeding site or moving for other reasons. Hence after any period on an infected plant, the aphids most likely to be infective with virus Y are those that have recently moved and made a brief feeding attempt.

TABLE I

INFECTIVITY OF *M. persicae* THAT HAD THEIR STYLETS INSERTED VARIOUS DISTANCES INTO TOBACCO PLANTS INFECTED WITH POTATO VIRUS Y

Time on infected plant, min.	Distance stylets inserted, μ	No. of aphids	Aphids infective, %
Under 1	Under 21	255	49
	Over 21	0	—
1–10	Under 21	139	23
	21–100	147	6
	Over 100	27	0
11–20	Under 21	39	12
	21–100	59	3
	Over 100	61	1

Penetrations Directly into Mesophyll

The foregoing results do not show whether aphids transmitted virus acquired from subepidermal tissues, for all of the aphids first inserted their stylets into infected epidermal cells. Tests were therefore made to learn whether subepidermal tissues contain virus Y that can be transmitted by *M. persicae*. A piece of upper epidermis was removed from an infected leaf by tearing across the leaf, picking up the epidermis near a torn vein, and pulling it away from the underlying mesophyll until the piece of epidermis ruptured. Sometimes an area of mesophyll up to a square centimeter was exposed. Single aphids were placed on the exposed mesophyll and observed with a hand lens. Though

most of them left the exposed mesophyll without attempting to feed, those that did were immediately tested for infectivity. Ten aphids were tested on five occasions. As controls, an equal number of aphids were tested after each made a brief attempt to feed on part of the leaf where the epidermis was intact. The numbers of aphids out of 50 that transmitted virus Y were 11 of those that inserted their stylets into the exposed mesophyll compared with 20 of the controls. This suggests that the mesophyll was a poorer source of virus than the epidermis. But removing the epidermis may have damaged the underlying mesophyll, which in turn may have affected the availability of virus to aphids. Therefore, a second experiment was made, similar to the first except that the control aphids made their feeding attempt into the piece of removed epidermis, which was stretched over moist blotting paper. This made the behavior of the control aphids similar to that of the others, for they too usually did not attempt to feed. However, aphids were placed on the piece of epidermis and the area of exposed mesophyll until 10 had attempted to feed on each. This experiment was repeated five times. The numbers out of 50 that transmitted virus Y from the piece of epidermis and the exposed mesophyll were 6 and 7 respectively. Thus the aphids became infective as readily when they made feeding attempts on exposed mesophyll as when they did so on the piece of removed epidermis. Conclusions from these results merit caution, however, because the few aphids that attempted to feed may not have been typical of the aphids as a whole.

Penetrations Through a Membrane into Infected Plants

It was found that the aphids insert their stylets through a membrane into tobacco leaves much more readily than they attempt to feed on mesophyll from which the epidermis has been removed. Moreover, aphids that did this could imbibe food. Therefore, experiments were made to learn whether aphids became infective when the stylets passed through virus-free material during the initial stage of penetration. Table II shows the results of five series of experiments in which aphids were tested for infectivity after they had inserted their stylets through virus-free membranes into infected plants. Only occasional aphids became infective. Yet over 50% of the control aphids invariably became infective when each made a brief feeding attempt on areas of the leaf not covered with the membrane. Aphids were allowed three to five minutes on the membranes, for they usually inserted their stylets through the membrane and superficially into the infected leaf during this time. They often had their stylets inserted over 50μ after five minutes on the membrane. In one experiment for example, each aphid was anesthetized after three to five minutes on a piece of healthy tobacco epidermis that was in contact with an infected leaf, and the distance the stylets were inserted was measured before the aphid was tested for infectivity. Thirty-five aphids inserted their stylets a mean distance of 28μ, compared with 33μ for an equal number of aphids that spent the same period on the leaf where it was not covered with a membrane. As the piece of healthy epidermis was not over 15μ thick, most

of the aphids inserted their stylets through it and superficially into the infected leaf. Yet not one of these aphids became infective, whereas 12 of the control aphids did.

In other tests, aphids were confined for hours and sometimes days on membranes that were in contact with infected leaves. Though these aphids had young and excreted, which shows that they fed, they were only occasionally infective when transferred to healthy test plants.

Also, penetration of the membranes did not reduce the infectivity of aphids any more than that of the epidermis of healthy tobacco plants. However, the first material tested as a membrane was embedding paraffin (melting point 43–45° C.). But these tests are not included, for the paraffin made aphids noninfective when they inserted their stylets into it, and this also greatly reduced the likelihood of an aphid becoming infective with virus Y during subsequent feeding attempts on infected leaves. These effects are worthy of further study.

TABLE II

EFFECT OF A VIRUS-FREE MEMBRANE OVER TOBACCO LEAVES INFECTED WITH POTATO VIRUS Y ON AVAILABILITY OF THE VIRUS TO APHIDS

Membrane	Aphids per test plant	Plants infected/No. tested	Percentage of control aphids infective after each briefly attempted to feed on infected plant without membrane
Tobacco epidermis	1	0/75	53
Onion epidermis	1	0/33	57
Tulip epidermis	1	0/40	60
Tulip epidermis	5	1/40	62
Parafilm	5	3/60	81

Penetrations into Infected Leaves Irradiated with Ultraviolet

Virus in the superficial tissues of a leaf can be inactivated by ultraviolet radiations (1, 7), and *M. persicae* feeds almost as readily on leaves so treated as on unirradiated leaves. Hence this offers another approach to the problem of whether aphids become infective with a virus *after* the stylets penetrate beyond the superficial tissues. Both surfaces of infected leaves were irradiated for 20 sec. at a distance of one inch from the surface of the tube of the ultraviolet lamp. The intensity of irradiation of 2537 Å on the surface of the leaf was about 10 milliwatts per square centimeter. The only visible effect of the irradiation was that some of the leaves became slightly bronzed about two days afterwards. To learn whether there was any virus available from the superficial tissues, aphids were tested for infectivity after periods of 30 sec. and 5 min. on the leaf immediately after irradiation. Also, single aphids were

anesthetized after 24 hr. on the irradiated leaf, and tested for infectivity if the stylets were inserted over 25μ, which was the approximate thickness of the epidermis. As controls, aphids were tested after the same periods on unirradiated leaves. Ten aphids were tested for each period and the experiment was repeated four times. The numbers of aphids out of 40 that became infective were as follows:

Time on infected leaf	30 sec.	5 min.	24 hr.
Leaf not irradiated	23	14	0
Leaf irradiated	0	0	0

Aphids that had their stylets inserted over 25μ after 24 hr. on irradiated leaves did not become infective. Yet the deeper tissues of irradiated leaves contained active virus, for sap extracts from them caused many lesions when rubbed on tobacco plants. But this result is perhaps not unexpected, for no transmissions occurred from aphids that had their stylets inserted into subepidermal tissues after 24 hr. on unirradiated leaves. The only aphids that became infective were those on unirradiated leaves for 30 sec. and 5 min. Hence, *M. persicae* failed to become infective with virus Y after the virus in the superficial tissues was inactivated by ultraviolet radiations.

Inoculation of Virus

The results of the following experiment show that *M. persicae* may be infective after the stylets penetrate subepidermal tissues of healthy plants. Aphids were transferred to a healthy tobacco plant immediately after each made a brief feeding attempt on an infected plant. Each aphid was anesthetized after three to five minutes on the healthy plant, and then tested for infectivity if the stylets were inserted over 25μ. Out of 100 aphids so tested, nine were infective with virus Y. These nine had their stylets inserted a mean distance of 88μ into the healthy plant.

Table III shows that infective aphids usually failed to transmit virus Y when they inserted their stylets through a membrane into healthy tobacco plants. Each aphid had first made a brief feeding attempt on an infected plant, during

TABLE III

EFFECT ON TRANSMISSION OF POTATO VIRUS Y WHEN INFECTIVE APHIDS INSERTED STYLETS THROUGH CERTAIN MEMBRANES INTO HEALTHY TOBACCO PLANTS
(five aphids per test plant)

Membrane	Time on first test plant	First test plants infected/No. tested	Second test plants infected/No. tested (no membrane)	% transmission by *single* aphids tested on plants without membrane
Tobacco epidermis	5 min.	0/7	—	80
Tulip epidermis	1 hr.	8/40	6/40	62
Parafilm	5 min.	3/40	38/40	87
Parafilm	1 hr.	2/20	11/20	70

which over 60% became infective. Groups of five of these aphids were confined on a membrane in contact with the leaf of a healthy test plant. After five minutes or one hour on the membrane, each group was transferred to a second test plant, where they fed directly into the plant for 24 hr. The greatest number of first test plants were infected when tulip epidermis was used as the membrane. But even these were many fewer than would have occurred in the absence of a membrane, for the aphids, being over 60% infective, should have infected the first test plants almost without exception. Other tests showed that aphids often retained their infectivity after the stylets pierced these membranes.

Discussion

There has never been any reason to conclude that aphids become infective with virus Y or transmit it other than during the initial stage of stylet penetration. The most likely interpretation of results presented here is that *M. persicae* usually does not become infective with virus Y or transmit it after the stylets penetrate beyond the first layer of cells. This is probably true of many aphid-borne viruses that are transmitted when their vectors make brief feeding attempts. That exceptions will be found seems likely, but they should be based on evidence, not on speculation.

A membrane over tobacco leaves infected with potato virus Y greatly reduced the numbers of aphids that became infective, as did also ultraviolet irradiation of leaves infected with virus Y (7), cabbage black ring spot virus, henbane mosaic virus, and sugar-beet mosaic virus (1). These four viruses are among those that are transmitted optimally by aphids that make brief feeding attempts after a period of not feeding. This marked reduction in availability of virus to aphids caused by membranes or ultraviolet irradiation appears to have the same basis. Ultraviolet irradiation inactivates all or most of the virus in the epidermal layer, and there is evidence that much of the virus in deeper tissues is not inactivated (1, 7). On irradiated leaves, therefore, aphids must insert their stylets through cells that contain little or no virus before they can become infective. The same is true when a virus-free membrane is placed over infected leaves (Table II).

Bawden, Hamlyn, and Watson (1) obtained more transmissions of henbane mosaic virus with aphids that spent 24 hr. on ultraviolet-irradiated leaves than with aphids that spent an hour. This was interpreted as evidence that some of the aphids became infective with virus from subepidermal tissues. The results with virus Y differ in that aphids did not become infective when they inserted their stylets into the subepidermal tissues of irradiated leaves. And aphids were rarely infective when their stylets were inserted into subepidermal tissues after several hours on infected tobacco plants (5). All viruses transmitted in this manner are probably not transmitted in exactly the same way, and henbane mosaic virus may differ from virus Y in that it is more often transmitted from subepidermal tissues. But if so, it also differs in another respect. Once the stylets penetrated beyond the epidermis,

aphids were more likely to be infective with virus Y the sooner the stylets were inserted into subepidermal tissues after a period of not feeding (Table I). And since many aphids insert their stylets into subepidermal tissues during the first hour, more would be infective with virus Y after an hour than after 24 hr. (other factors being the same). But this was not so with henbane mosaic virus (1).

Bawden *et al.* (1) presented their results with ultraviolet irradiation as supporting evidence for an hypothesis they postulated as follows: "The correlation between feeding on the epidermis and the numbers of transmissions is more plausibly explained simply by postulating that the epidermal cells contain more extractable virus than the deeper tissues. The stylets of aphids filled with sap taken from the epidermis would be more likely to include virus than when they contain sap from other tissues". They assume that aphids become infective with such viruses after the stylets penetrate beyond the epidermal layer, an assumption not justified in view of the results with virus Y. That there is more to this type of transmission than concentration of virus in the epidermis is shown by the fact that aphids rarely became infective with virus Y when they inserted their stylets through a membrane into the epidermis of infected plants (Table II). Also, aphids became infective with virus Y as readily when they inserted their stylets *directly* into mesophyll cells beneath the epidermis as when they inserted their stylets into the piece of removed epidermis. Their main evidence that epidermal cells contain more extractable virus than other tissues is that sap from unirradiated leaves caused about four times as many infections as that from irradiated leaves. To conclude that epidermal cells contain more virus than other tissues, they assumed that ultraviolet irradiation inactivated *only* virus in the epidermis. This assumption has since been questioned by Benda, who found that considerable ultraviolet radiation passes through the epidermal layer (3). Moreover, it is questionable whether the availability of virus to aphids is related to the infectivity of sap extracts. For example, severe etch virus is much more concentrated in sap extracts of infected tobacco than is a related strain, mild etch virus (2); yet *M. persicae* transmits mild etch virus as readily from tobacco as it does severe etch virus (11).

When green peach aphids are placed on a host plant after a period without food, each one usually makes a few brief attempts to feed before it settles at one site (4). Only through aphids becoming infective with virus Y, or a similarly transmitted virus, is it known that they acquire something from plants during these first attempts to feed. And since such aphids can immediately infect susceptible plants, some of this acquired material must pass from the aphid to subsequent feeding sites. The quantity of material acquired at such times appears to be too small to be important as food (9, 12). At present, this material can only be studied indirectly through the transmission of a virus. Studies on the aphid transmission of virus Y have shown that some of this acquired material is carried near the tip of the stylets (8). What is its function? Can it be part of a mechanism that enables an aphid to test the

suitability of the site? If so, what is the nature of a mechanism that enables an aphid to reject a site merely by inserting its stylets part way through the epidermis when it imbibes its food from deeper tissues? To conclude that this type of transmission is fortuitous would be premature. And too little is known about it to postulate generalized explanation with confidence. More data are obviously needed. However, further studies of this type of transmission promise a better understanding of the *feeding* of aphids as well. The converse, of course, is also true.

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STUDIES ON THE TRANSDUCTION PHENOMENON

I. PRACTICAL APPLICATIONS IN THE LABORATORY[1]

By W. R. Bailey[2]

Abstract

Transduction has a practical application in a laboratory where the serological analysis of *Salmonella* organisms is carried out. Non-motile variants of strains may be treated with transducing phage, such that they develop flagella and motility. Such treated strains may then be typed with the appropriate antisera. Non-motile, poorly flagellated strains of *S. derby*, *S. dublin*, and *S. typhi* Vi were successfully transduced to motile forms. A phage isolated from *S. thompson* strain 1/19 (Williams Smith) was found to possess transducing properties. Laboratory methods for the transducing procedure are outlined.

Three years ago a new scientific term, 'transduction', was introduced by Zinder and Lederberg (13) to define a phenomenon whereby nutritional, fermentative, or antigenic characters could be transferred or literally led across by *S. typhi-murium* phage PLT22 from one bacterial cell to another. Since the classical work of these investigators on genetic exchange in *Salmonella*, others have demonstrated the capacity of *Salmonella* phages to transduce a varied number of characters. Iseki and Sakai (7, 8) successfully transformed group E_1 salmonellae (somatic complex '3, 10') to group E_4 (somatic complex '3, 15') and transferred drug resistance and fermentative properties with phage lysates. Stocker, Zinder, and Lederberg (11) presented strong suggestive evidence that a number of genetic traits were involved in the expression and suppression of motility, while Baron, Formal, and Spilman (1), using *S. typhi* Vi phage, induced streptomycin resistance, fermentative and antigenic changes in *S. paratyphi* C.

The basic requirements for successful transduction have now been clearly established. The donor cell, the cell in which the phage is propagated, must exhibit a sensitivity to the phage, while the recipient, the cell undergoing treatment, must at least possess the ability to adsorb the phage. It has also been established that the factor which determines the sensitivity to or the adsorption ability for the phage is some receptor site common to both donor and recipient. This receptor site for the *S. typhimurium* phage has been identified as somatic antigen '12'. It has been the experience of other investigators in the field, as well as the author, that strains lacking this antigen are unable to function as donors or recipients.

The possession of somatic antigen '12', however, by any given strain does not necessarily identify it as an efficient donor or recipient in transduction. The inability of a cell to donate or receive may be due to the presence in it

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Contribution from the Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Ontario.
[2]*National Salmonella Typing and Reference Centre, Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Ontario. Present address: Department of Biological Sciences, University of Delaware, Newark, Delaware, U.S.A.*

of a prophage which is related to the transducing phage, thus preventing the entry of the latter into the cell. Failure in transduction may also be due to a non-homologous relationship between donor genetic material and recipient replaceable material. Both of these become limiting factors in transduction experiments.

It has been shown by Lederberg and Edwards (10) and other investigators that a number of antigenic hybrids can be produced in the laboratory through transduction, and non-motile variants can be transduced to motile forms. This capacity of a transducing phage for inducing motility has been shown to have a practical application in a laboratory where the serological analysis of *Salmonella* types is carried out. Occasionally non-motile salmonellae, other than *S. gallinarum-pullorum*, are received for identification and such strains cannot by typed unless the H antigens are identifiable. Some such strains are true O forms, whereas others possess a few flagella which have lost their motory function and have been referred to as paralyzed strains.

Methods for Inducing Motility in Non-motile Forms

Preparation of Lysate

Place about 0.05 ml. of a 24-hr. broth culture of the selected donor strain in 5 ml. fresh meat infusion broth in a 15 ml. centrifuge tube to which is added a small loopful of phage. The ratio of phage to donor cells should be about 1 : 10. Too high a ratio of phage to cells will inhibit lysis (Boyd (2)). Incubate the mixture in a 37° C. water bath so that about one-third to one-half of the liquid in the tube is submerged. This will set up convection currents which will bring cells and phage particles in frequent contact. After four to five hours, or preceding the termination of the logarithmic phase of growth of the cells, remove the mixture from the water bath and centrifuge it at 2500 r.p.m. for 30 min. Decant the supernatant into a test tube, close the tube tightly with a rubber stopper, and completely immerse it in a 60° C. water bath for 30 min. to destroy all viable cells remaining in the lysate.

Transduction Procedure

Suspend about 10^9 to 10^{10} cells from a 24 hr. agar culture of the strain to be transduced in 1.0 ml. of meat infusion broth or 0.01 *N* $MgCl_2$ solution. Add 1.0 ml. of the sterile fresh lysate, mix thoroughly, and allow adsorption to proceed at 37° C. in a water bath for 20–30 min. Throw down the infected cells by centrifugation at 2500 r.p.m. for 20 min., decant, and discard the supernatant. Resuspend the cell sediment in the small volume of menstruum remaining in the tube and spread this concentrated suspension out X-wise on the dry surface of semisolid agar (0.3–0.4% agar) in a Petri plate (see Plate I). It is advisable to break the surface of the agar to arrest the spread of the cell suspension. Incubate the inoculated plate in a level position at 37° C. with the lid partly supported on small corks for 12–16 hr. This ventilation will prevent condensation on the under surface of the lid.

PLATE I

FIG. 1. Swarms developing from a formerly non-motile population after phage treatment.

Examine the plate for swarms which appear as confluent outgrowths in the medium in about 12 hr. Hanging-drop preparations show these swarms to be comprised of actively motile forms.

Results

The H antigens identified in these actively motile forms are usually those exhibited by the original motile parent from which the variants were derived. The type identity can be determined from the results obtained, when the H antigenic structure of the donor is known and the bacteriological sterility of the lysate established. Should there be any relationship between the H antigens of the transduced form and those of the donor cell, another antigenically unrelated strain may be used as donor to confirm the findings. This will eliminate the possibility of the carryover of antigenic determinant as opposed to motile determinant.

In the past year, non-motile strains of groups B, C, and D of the *Salmonella* genus have been received for type identification. The Group B and D strains were treated successfully with *S. typhi-murium* phage PLT22 using *S. typhi-murium* str. TM_2 as donor, while the Group C non-motile form was treated with a phage isolated from a lysogenic strain of *S. thompson*, str. 1/19 of Williams Smith (12), propagated on either *S. cholerae-suis* or *S. bareilly*. Table I shows the results obtained. A lysate of the *S. dublin* non-motile strain transduced the *S. derby* non-motile strain, but the former could not be transduced by a lysate of the latter. An O form of *S. typhi-murium*, on the other hand, was quite efficient as a donor in the transduction of the *S. dublin* strain. Similarly the *S. dublin* strain when used as donor was effective in the transduction of the non-motile *S. typhi* Vi strain.

TABLE I

INDUCTION OF MOTILITY BY PHAGE IN NON-MOTILE SALMONELLAE

Phage	Donor	Recipient	Result	Type
PLT 22	TM_2 (4, 5, 12; i, -, 1, 2)	Str. 70690 4, 12; -,-	4, 12; f, g, -	*S. derby*
PLT 22	Str. 72202 1, 9, 12; -,-	Str. 70690 4, 12; -,-	4, 12; f, g, -	*S. derby*
PLT 22	TM_2 (4, 5, 12; i, -, 1, 2)	Str. 72202 1, 9, 12; -,-	1, 9, 12; g, p, -	*S. dublin*
PLT 22	Str. 70690 4, 12; -,-	Str. 72202 1, 9, 12; -,-	No swarms	
PLT 22	*S. typhi-murium* O form (4, 5, 12; -,-)	Str. 72202 1, 9, 12; -,-	1, 9, 12; g, p	*S. dublin*
PLT 22	TM_2 (4, 5, 12; i, -, 1, 2)	Str. 71905 9, 12, Vi; -,-	9, 12, Vi; d, -	*S. typhi*
PLT 22	Str. 72202 1, 9, 12; -,-	Str. 71905 9, 12, Vi; -,-	9, 12, Vi; d, -	*S. typhi*
P 1/19	*S. cholerae-suis* C_2 6, 7; c, -, 1, 5	Str. 550396 6, 7; -,-	6, 7; c, -, 1, 5	*S. paratyphi* C W form
P 1/19	*S. bareilly* B_2 6, 7; y, -, 1, 5	Str. 550396 6, 7; -,-	6, 7; c, -, 1, 5	*S. paratyphi* C W form

Origin and Characters of the Strains

The strain of *S. derby* was isolated from the liver and small intestine of two diseased turkeys. The non-motile *S. dublin* strain was represented by four cultures isolated from the urine, stool, and blood of an adult male at various stages of illness. Whereas the urine and stool isolates gave rise to colonies of normal appearance, the blood isolates produced large mucoid slimy colonies that tended to coalesce. The 'normal' strains were readily transduced to motile forms by PLT22 phage while the mucoid forms showed no response. Water and broth extracts of the slime or mucus exhibited no phagicidal activity and the mucoid strain itself adsorbed the phage with the same avidity as did the non-mucoid strain. The slime layer showed a strong relationship to *Klebsiella* type 13 (P. R. Edwards, personal communication). Non-mucoid variants of the mucoid strain responded readily to transduction. Interestingly enough the identification of these isolates as *S. dublin*, with the aid of transduction, represents the first report of this serotype in humans in Canada.

The *S. typhi* Vi non-motile strain (str. 71905) was one of two cultures isolated from a man and his wife in Nova Scotia, the former being a carrier and the latter a clinical case of typhoid. While one might infer the serotype of an organism with a somatic structure of 9, 12, Vi, since no presently known type other than *S. typhi* possesses this complex, the strain did offer a convenient index in the study of transduction. Furthermore, it showed that the Vi antigen did not interfere with the phenomenon since motility could readily be transduced in these non-motile strains. Both cultures were phage typed as B_3.

The three non-motile strains *S. derby*, *S. dublin*, and *S. typhi* would not agglutinate in H antisera but yet flagella staining techniques (Gray (5)) revealed that a very small percentage of the population possessed well-developed flagella. These appendages, however, had lost their locomotory properties and the flagellated forms were too few in number to give visible H agglutination. Absorption experiments with the untreated *S. typhi* strain indicated that it was only capable of removing an almost negligible amount of H agglutinins from an anti 'd' serum.

The *S. paratyphi* C non-motile W form was obtained from pus on the leg of a patient with osteomyelitis of the tibia. The transduced motile form was in phase 1, 'c'. The second phase, '1, 5', developed only after cultivation on semisolid agar plates containing 'c' antiserum. Its identity as *S. paratyphi* C was confirmed by biochemical studies.

Discussion and Conclusions

The author believes that transduction can be employed as a useful tool in a laboratory where the serological analysis of *Salmonella* strains is carried out. *S. typhi-murium* phage PLT22 will induce motility in members of

Groups A, B, and D of the genus. Transduction of *S. paratyphi* A O form to a motile form has been successfully carried out with PLT22 phage grown on *S. typhi-murium* str. TM_2 (unpublished data).

S. thompson phage P 1/19 has exhibited a similar property for at least one serotype in group C and judging from the number of lysogenic salmonellae, known or suspected, it is quite likely that other phages may be found which have this transducing capacity. A set of such phages could be used as a laboratory aid in the serodiagnosis of non-motile salmonellae.

The transduction of non-motile forms using lysates of O forms has been previously discussed by Stocker *et al.* (11) and will not be discussed here. This report, however, shows also that paralyzed forms may be used as donors and it is surmised that the same principles are involved. The unilateral transfer of material between *S. dublin* and *S. derby* was an interesting example of one of the complexities of the phenomenon which have not as yet been elucidated.

Non-motile flagellated salmonellae have been previously reported: *S. aberdeen* (Kauffmann (9)); *S. san-diego* (Edwards, Moran, and Bruner (3)); *S. paratyphi* B (Hirsch (6)); *S. typhi-murium* (Friewer and Leifson (4)). This investigation has revealed that there are also such strains of *S. derby*, *S. dublin*, and *S. typhi* Vi. Unlike the *S. paratyphi* B strain of Hirsch, which had well-developed flagella, examination of broth cultures of these three strains revealed that only a few cells were flagellated.

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STUDIES ON THE TRANSDUCTION PHENOMENON

II. THE OCCURRENCE OF SALMONELLA TRANSDUCING PHAGES IN NATURE[1]

By W. R. Bailey[2]

Abstract

An investigation with 115 strains of *S. typhi-murium* isolated from a variety of sources revealed that 12.1% (or 14 strains) carried transducing phage. The highest incidence was found among strains obtained from human sources. Suggestive evidence of antigenic changes taking place in nature is given and the possible role of transduction in the evolution of the genus *Salmonella* is discussed.

Several investigators have shown that bacteriophages isolated from lysogenic salmonellae can induce antigenic changes in other *Salmonella* strains in the laboratory to produce hybrid forms. Some of these hybrid forms have the antigenic structure of officially recognized serotypes, while others through 'directed mutation' exhibit the antigenic structure of types hitherto undiscovered in nature. It is justifiable, in the face of past and present trends, to assume that if such types had been isolated in nature they would have been recognized as new types of *Salmonella* and accorded a name in the Kauffmann–White Schema. It has also been suggested that transduction in nature may be partly responsible for the multiplicity of *Salmonella* types, as well as being partly responsible for the appearance of new types in various parts of the world.

Because of these suggestions a recent survey was started to investigate the distribution of transducing phages in nature. The scope of this investigation was relatively narrow in that it was limited to a study of phages isolated from lysogenic strains of *S. typhi-murium* occurring in Canada and submitted to the *Salmonella* center for identification.

Methods

Determination of Lysogeny

The basal or indicator strain used was *S. typhi-murium* str. TM_2 (Lederberg). Smooth colonies of both the indicator strain and the strain being investigated were seeded to 3 ml. of meat infusion broth and incubated in a 37° C. water bath for six hours with the water level so adjusted as to set up and maintain convection currents in the tube. The mixed culture was placed at 5° C. overnight and finally centrifuged at 2500 r.p.m. for 30 min. The supernatant was decanted and heated at 60° C. for 30 min. One loopful of a young broth culture (four hours) of each strain was then spread on the dry surface of an agar plate to cover two discrete circular areas of approximately 3/4 in. diameter. These were allowed to dry and a loopful of the heated lysate applied to each

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[2]*National Salmonella Typing and Reference Centre, Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Ontario. Present address: Department of Biological Sciences, University of Delaware, Newark, Delaware, U.S.A.*

area according to the method of Fisk (3). After the lysate dried out, the plates were placed at 37° C. for three to four hours until plaques were discernible, then left at room temperature overnight. Lysogeny was determined from the appearance of plaques on the strain TM_2. The temperate phage was purified by picking a plaque to a small volume of broth, allowing the phage to propagate, centrifuging, heating, and finally seeding to broth inoculated with the basal strain.

Determination of Transducing Ability

The indicator strain employed was a non-motile Vi strain of *S. typhi*, str. *1*1905, as this culture has repeatedly failed to produce any spontaneous motile mutants. The transducing procedure used was the same as that used in Part I of this report (p. 549). Positive transduction was recorded if the test strain developed active swarms on semisolid agar after phage treatment and if the phage free control showed no change.

Each phage isolated from a lysogenic strain of *S. typhi-murium* which was capable of transducing motility was also tested for its ability to transduce phase 1 H antigen "i" from the donor *S. typhi-murium* (4,5,12; i,–,1,2) to *S. paratyphi* B (4,5,12; b,–,1,2) so that the latter or recipient strain exhibited the antigenic structure of *S. typhi-murium* (4,5,12; i,–,1,2).

Source of *S. typhi-murium* Strains Tested

Cultures isolated from humans, chickens, turkeys, ducks, mice, rabbits, cattle, guinea pigs, and a crow were examined. A total of 115 strains were tested and 81 (or 61.7%) of these were lysogenic for a phage active on *S. typhi-murium* str. TM_2. Fourteen strains (12% of the strains examined) were found to be carrying transducing phage. Of these 14 strains, nine were of human origin, four were from poultry, and one was from a bovine source. Table I shows the distribution in four provinces of Canada of strains carrying transducing phage. The findings seem to indicate that there is a higher incidence of transducing phages in strains of human origin than in those from other sources.

TABLE I

SOURCE AND INCIDENCE OF TRANSDUCING PHAGES AMONG STRAINS OF *S. typhi-murium* ISOLATED IN CANADA

British Columbia		Alberta		Saskatchewan		Quebec	
Human	Other	Human	Other	Human	Other	Human	Other
2	0	2	5	3	0	2	0

NOTE:
Total number of strains examined = *115*
Number of human strains examined = *56*
Number of non-human strains examined = *59*
Number of transducing phages from human strains = *9*
Percentage of human strains with transducing phage = *16*
Number of transducing phages from non-human strains = *5*
Percentage of non-human strains with transducing phage = *8.4*

The failure to find transducing phage in the non-human isolates from British Columbia, Saskatchewan, and Quebec may be due to the fact that there were fewer cultures of non-human origin examined from those provinces than from Alberta.

Discussion

There is little doubt that phages which possess transducing capacity are fairly widely distributed in nature. It has been shown that of a number of *S. typhi-murium* strains examined from various sources 12.1% of them carried phages possessing this property. The percentage of human strains carrying such phages is even higher. Edwards, Davis, and Cherry (2) have recently reported the presence of transducing phages in lysogenic strains of Groups C, D, E, and G of the genus. These were capable of transferring antigenic characters between members of their respective groups. The work of Iseki and Sakai (6, 7) with *S. newington* phage and of Baron, Formal, and Spilman (1) with *S. typhi* phage E_1 gives support to the claim of genus-wide distribution of such phages.

The suggestion, however, that transducing phage is playing a major role in the development of the immunologically complex *Salmonella* serotypes occurring in nature is still based on conjecture. On the other hand one cannot afford to be dogmatic and claim this not to be so, although it must be admitted that there is no concrete but only suggestive evidence of this. We have no definite evidence that listed serotypes remain stable in nature although outbreaks of gastroenteritis invariably indicate a constancy of antigenic pattern in the pathogen involved. In such instances, the epidemiological value of serologic typing has been convincingly demonstrated.

The sudden and unexplained appearance of new serotypes in certain parts of the world, however, are suggestive of changes occurring in nature. In Canada, each year for the past several years, types hitherto not known in this country have been suddenly 'discovered', usually among the human population but occasionally in the lower animals. Some of these make a single appearance while others persist. In most instances the appearance of these types has been quite unexplainable epidemiologically. The great majority of these are members of somatic groups from which transducing phages have been obtained.

Certain isolates from cases of active infection exhibit the antigenic formulae of recognized pathogens and yet do not fit the biochemical pattern of their type. The clinical syndrome, moreover, initiated by the infection does not conform with that usually encountered with the indicated pathogen. One example of this is an organism which is *d*-tartrate positive and has the antigenic structure of *S. paratyphi* B and, moreover, produces gastroenteritis rather than paratyphoid fever. An outbreak of gastroenteritis in a children's home in England (reported by Gray and Harvey (5)), 43 cases of gastroenteritis and 56 symptomless excreters in various parts of Britain reported by McDonald, Price, and Taylor (8), and a further outbreak of an alimentary infection reported by Grant and Norton (4) all involved an organism with the antigenic

structure of *S. paratyphi* B in phase 1 (4,5,12; b,–). The isolates were culturally and biochemically unlike this pathogen and could not be phage typed as *S. paratyphi* B. Such a strain has also been reported by Kauffmann in both the monophasic and diphasic state. In Canada we also have found monophasic and diphasic strains of a *d*-tartrate positive organism antigenically similar to *S. paratyphi* B, which was involved in infections of gastroenteritis. The English authors have felt that this is a new enteric pathogen and Kauffmann has suggested the name of *S. java* for this type. The author agrees with Kauffmann's suggestion that '*S. java*' might be a serotype engendered by transduction in nature.

Moreover, on occasion we have identified as *S. typhi-murium* (antigenically) isolates from fatal cases of septicemia in Canada. One Provincial Department of Health isolated an organism serotyped as *S. typhi-murium* from a known *S. paratyphi* B carrier. Transduction of *S. paratyphi* B to *S. typhi-murium* can be so easily demonstrated in the laboratory that it seems at least possible that the strains identified serologically as *S. typhi-murium* in these instances might be transduced forms of *S. paratyphi* B.

We do not have sufficient evidence at this time to state positively that transduction and/or related phenomena are playing some definite part in the evolution of the *Salmonella* genus, but we must at least keep an open mind on the subject and admit this to be a possibility.

The serological analysis of the salmonellae and other members of the Enterobacteriaceae has been and continues to be a very useful tool in the laboratory, but if evidence of the instability of types in the host and of the evolvement of new types in nature is forthcoming, one shall have to use additional criteria in our diagnostic procedures when they are applied to epidemiological investigations.

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UTILIZATION OF ADDED SUBSTRATES BY UREDOSPORES OF WHEAT STEM RUST[1]

By P. Shu, A. C. Neish, and G. A. Ledingham

Abstract

Uredospores of wheat stem rust utilized a number of externally-supplied, labelled carbohydrates, amino acids, and volatile fatty acids. The carbon of these substrates appeared in the spore materials and in the carbon dioxide. This metabolic activity, though very weak, is definite. D-Mannose, D-mannitol, D-glucose, sucrose, and D-fructose were utilized better than the other carbohydrates. Glutamic acid gave the highest yield of carbon dioxide while the basic amino acids, L-arginine and L-lysine, were more efficiently incorporated into the spore material.

Resting spores generally exhibit distinct but very weak respiratory activity. The germination process generates strong enzymatic activities and results in a high rate of metabolism, sufficient to sustain the growth process, when substrates are supplied continuously. The uredospores of wheat stem rust are unusual, however, in that the respiration rate is not changed significantly by germination (6). During germination the lipid and protein fractions of the spores are utilized but no consumption of externally supplied substrates was observed by the manometric technique (6). More recent investigations (5) indicate that enzymes related to the metabolism of phosphorylated carbohydrates are present although the spores do not utilize their large reserves of mannan. This suggests that the spores do not have complete facilities or enzyme systems for the utilization of substrates which are metabolized by non-obligate parasites as energy and carbon sources. Therefore, to determine whether externally supplied substrates are consumed by the spores, various compounds labelled with C^{14} were supplied and the results are reported below.

Methods

Eight milligrams of C^{14}-labelled substrate (approximately 150–300 mμc.) dissolved in 0.8 ml. of distilled water were poured on the surface of 2% agar in a Petri dish (diameter 9 cm.) containing 100 p.p.m. aureomycin hydrochloride and 100 p.p.m. actidione. No growth of contaminants was observed in the presence of these antibiotics. The substrate was thoroughly impregnated into the surface layer of the agar by being heated under an infrared lamp (50° C.) for 45 min. All materials were sterilized either in an autoclave at 125° C. for 20 min. or through a Seitz filter. The plate was then inoculated evenly with 40 mgm. of wheat stem rust uredospores (strain 15B) and then inverted over its cover, which contained 10 ml. of 0.2 *N* carbonate-free sodium hydroxide solution. The plate was supported by two thin glass rods set inside the Petri dish cover to leave a small opening for free exchange of air with the surrounding atmosphere. The opening was sufficiently close to the

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surface of sodium hydroxide solution so that the carbon dioxide produced was completely absorbed. The Petri dish thus prepared was enclosed in a 4 in. × 7.5 in. (diam.) crystallization dish and allowed to stand at 25° C. for 16 hr. After this period of incubation the sodium hydroxide solution was quantitatively transferred to a combustion flask with a hypodermic syringe. The spore material was quantitatively scraped off from the plate with a rubber policeman and washed four times with 80 ml. of hot Vel detergent solution (0.1%, 70° C.) and finally rinsed with 80 ml. of distilled water. It was then converted to carbon dioxide with chromic acid combustion fluid for C^{14} measurement. The C^{14} determination was made by the method of Buchanan and Nakao (2). Duplicate experiments were run on each substrate.

With actidione present no germination occurred under these experimental conditions. However, germination is not essential for this study since the rates of oxygen consumption and carbon dioxide evolution are not appreciably changed by the process of germination. Furthermore in preliminary experiments, it was found possible to vary the extent of germination by changing the relative distance between the sodium hydroxide solution and the spore plate when incubation was carried out in a closed jar. This effect was probably due to the removal of varying amounts of the "self-inhibition" factor described by Allen (1). The results of such experiments with labelled substrates, e.g. glucose, showed no significant differences between germinated and

TABLE I

UTILIZATION OF SUGARS BY UREDOSPORES OF WHEAT STEM RUST

	μM. of labelled carbon of substrate converted into:		% of total labelled carbon found in:		% of total carbon derived from labelled carbon	
Labelled substrate	Respiratory carbon dioxide	Spore carbon	Respiratory carbon dioxide	Washed spores	Respiratory carbon dioxide	Spore carbon
Sucrose[1]	3.40	2.92	1.18	1.01	7.40	0.45
	3.20	3.31	1.13	1.18	7.50	0.39
D-Fructose[1]	2.60	2.01	0.97	0.75	4.40	0.29
	2.20	2.57	0.80	0.97	6.00	0.34
D-Glucose[1]	4.90	2.81	1.83	1.05	7.90	0.42
	3.90	2.57	1.47	0.97	8.10	0.37
D-Glucose-1-C^{14}	1.30	0.50	2.91	0.97	3.20	0.08
	1.30	1.04	2.88	0.82	2.90	0.08
D-Glucose-2-C^{14}	0.47	—	1.06	—	1.40	—
	0.48	—	1.08	—	1.30	—
D-Mannose-1-C^{14}	1.30	0.35	3.03	0.83	2.80	0.06
	—	0.49	—	1.02	—	0.07
D-Allose-1-C^{14}	0.09	0.006	0.19	0.01	0.07	0.001
	0.03	0.005	0.07	0.01	0.07	0.001
D-Sedoheptulose-2-C^{14}	0.04	0.04	0.10	0.10	0.07	0.01
	0.04	0.03	0.11	0.09	0.26	0.01
D-Ribose-1-C^{14}	0.41	0.16	0.76	0.28	1.03	0.03
	0.37	0.18	0.70	0.31	0.82	0.03
D-Xylose-1-C^{14}	0.13	0.08	0.25	0.16	0.24	0.01
	0.14	0.06	0.28	0.12	0.28	0.01

[1]*Uniformly labelled.*

ungerminated spores in the average yields of total and active carbon dioxide produced during the incubation period. This also indicates that the metabolic pattern is not significantly affected by germination.

Results and Discussion

When C^{14}-labelled compounds were supplied to the uredospores of wheat stem rust as substrates, in most cases active carbon appeared in respiratory carbon dioxide and spore material. The rates of such conversions varied with different substrates.

Among the sugars tested, mannose, glucose, sucrose, and fructose gave highest yields of carbon dioxide (Table I). Ribose was converted somewhat better than xylose or sedoheptulose. An unnatural sugar, allose, was not significantly metabolized. It was noted that more C-1 of glucose appeared in carbon dioxide than any other carbons of the molecule. This suggests that some carbon dioxide was produced by initial oxidation of glucose to gluconate followed by decarboxylation. The enzymes involved in these reactions have been demonstrated with the cell-free extracts of the rust spores (5). It was noted also that mannose was actively metabolized yet a large reserve of mannan in spores was not utilized, indicating the probable absence of mannanase activity.

These uredospores were capable of utilizing sugar alcohols (Table II). Mannitol gave the best results although, unlike glucitol, this alcohol is not utilized by higher plants. Glycerol and arabitol were also utilized and the alcohols have been found in spore extracts in considerable amounts (3, 4). Arabitol probably is derived from reduction of ribulose by reduced diphosphopyridine nucleotide and the enzyme system catalyzing such a reaction has been demonstrated with a cell-free preparation of the spores (4).

The extent of incorporation of carbon from sugars and their alcohols into the spore material was roughly parallel to the amount oxidized to carbon dioxide (Tables I and II).

TABLE II

UTILIZATION OF SUGAR ALCOHOLS BY UREDOSPORES OF WHEAT STEM RUST

Labelled substrate	μM. of labelled carbon of substrate converted into:		% of total labelled carbon found in:		% of total carbon derived from labelled carbon	
	Respiratory carbon dioxide	Spore carbon	Respiratory carbon dioxide	Washed spores	Respiratory carbon dioxide	Spore carbon
Glycerol-1-3-C^{14}	0.37 0.41	0.51 0.44	0.29 0.28	0.21 0.23	0.89 0.96	0.07 0.07
D-Arabitol-1-C^{14}	0.70 0.69	0.47 0.58	0.90 1.10	1.32 1.30	1.03 1.38	0.07 0.08
D-Mannitol-1-6-C^{14}	2.70 2.70	1.00 0.86	1.10 0.97	3.00 3.01	5.21 4.59	0.16 0.15
D-Glucitol-1-C^{14}	0.30 0.43	0.06 0.14	0.20 0.27	0.66 0.95	1.53 1.05	0.01 0.02

TABLE III

UTILIZATION OF VOLATILE FATTY ACIDS BY UREDOSPORES OF WHEAT STEM RUST

Labelled substrate	μM. of labelled carbon of substrate converted into:		% of total labelled carbon found in:		% of total carbon derived from labelled carbon	
	Respiratory carbon dioxide	Spore carbon	Respiratory carbon dioxide	Washed spores	Respiratory carbon dioxide	Spore carbon
Na formate-C^{14}	0.45	0.08	0.38	0.06	0.44	0.01
	0.45	0.06	0.38	0.03	0.52	0.01
Na acetate ($C^{e}H_3$ labelled)	0.95	—	0.97	—	1.86	—
	1.02	—	1.05	—	1.78	—
Na acetate ($C^{e}OOH$ labelled)	1.34	0.23	1.35	0.24	2.00	0.04
	1.58	0.28	1.61	0.30	2.68	0.04
Na propionate ($C^{e}OOH$ labelled)	1.59	0.07	1.84	0.10	0.31	0.01
	1.60	0.07	1.89	0.11	0.23	0.01

TABLE IV

UTILIZATION OF AMINO ACIDS BY UREDOSPORES OF WHEAT STEM RUST

Labelled substrate[1]	μM. of labelled carbon of substrate converted into:		% of total labelled carbon found in:		% of total carbon derived from labelled carbon	
	Respiratory carbon dioxide	Spore carbon	Respiratory carbon dioxide	Washed spores	Respiratory carbon dioxide	Spore carbon
L-Glutamic acid	7.87	0.17	2.90	0.06	13.0	0.02
	6.49	0.28	2.39	0.10	9.24	0.03
L-Aspartic acid	1.78	0.15	0.74	0.06	3.46	0.02
	1.75	0.14	0.73	0.06	3.07	0.02
L-Isoleucine	0.82	0.41	0.22	0.11	2.93	0.06
	0.88	0.34	0.24	0.09	3.22	0.05
L-Leucine	1.28	0.31	0.35	0.08	2.27	0.04
	1.45	0.32	0.39	0.09	2.13	0.05
-Arginine	0.84	1.83	0.30	0.66	2.47	0.03
	0.68	1.24	0.25	0.45	0.97	0.02
L-α-Alanine	0.33	0.09	0.12	0.03	1.65	0.01
	0.25	0.10	0.09	0.04	1.60	0.01
L-Phenylalanine	0.20	0.30	0.04	0.07	0.67	0.05
	0.17	0.35	0.04	0.08	1.17	0.05
Glycine	0.35	0.21	0.16	0.10	0.69	0.03
	0.41	0.26	0.19	0.12	0.79	0.04
L-Threonine	0.11	0.16	0.04	0.06	0.34	0.03
	0.13	0.13	0.05	0.05	—	0.02
L-Proline	0.24	0.79	0.07	0.23	0.44	0.13
	0.20	0.40	0.06	0.12	0.72	0.15
L-Lysine	0.12	1.47	0.04	0.44	—	0.20
	0.21	1.60	0.06	0.48	0.35	0.21

[1]*Uniformly labelled.*

Data summarized in Table III showed that formate was neither dehydrogenated nor absorbed by the cells. A higher proportion of carboxyl carbon of acetate than methyl carbon was converted to carbon dioxide. Propionate was decarboxylated by the spores.

Glutamic acid gave the highest yield of carbon dioxide of any substrate (Table IV). Aspartic acid, isoleucine, leucine, alanine, arginine, and glycine gave fair yields and phenylalanine, threonine, proline, and lysine gave poor yields of carbon dioxide. The mode of amino acid metabolism seems to be different from that of carbohydrates. Glutamic acid, the best carbon dioxide producing substrate, was incorporated into the cell to the same extent as some other amino acids such as aspartic acid, leucine, isoleucine. On the other hand, acids which were comparatively poor carbon dioxide producers, such as threonine, proline, phenylalanine, and lysine were assimilated into the cell to a considerable extent. The basic amino acids, arginine and lysine, were the most readily assimilated of all the amino acids tested.

These results indicate that the uredospores of wheat stem rust have complete enzyme systems for the utilization of carbohydrates, lipids, and amino acids. However, carbon dioxide derived from externally-supplied substrates accounts for a very small fraction of the total, while the endogenous substrates were the dominating source of respiratory carbon dioxide. It is obvious that the magnitude of substrate utilization is not sufficient to sustain a vigorous autocatalytic growth process. Therefore, if the germination process cannot produce these enzyme systems in quantity, growth cannot be carried beyond these initial stages. It would be interesting to know whether fully developed rust mycelium differs in any significant features from germinating spores in its ability to utilize exogenous substrates. Work is now in progress in this laboratory using both uredospores and "tufts" of mycelium of flax rust which have been produced under aseptic conditions on leaf tissue cultures. This avoids the necessity of using antibiotics to control growth of contaminants and, it is hoped, will provide further insight into the metabolic processes of these obligate parasites.

Acknowledgment

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A STUDY OF THE INTRANASAL CHALLENGE ASSAY OF PERTUSSIS VACCINE USING IODINE-131[1]

By J. E. Logan, B. W. Griffiths, M. A. Mason, and L. Greenberg

Abstract

The present study was undertaken to determine the cause of the wide variation in the response of mice following intranasal challenge with virulent *H. pertussis*. Mice, both normal and immunized, were inoculated intranasally with killed *H. pertussis* organisms labelled with iodine-131. The course of the tagged antigens, in the lungs and other organs of these animals, was followed by radioisotope counting. The lung uptake varied from 31.6% to 77.5% of the challenge dose in immunized animals and from 25.0% to 83.7% in non-immunized animals. It was considered that this variation was large enough to contribute substantially to the wide discrepancies found in animal assays. To date the cultures employed for intranasal challenge have all been of low virulence when administered by this route. A considerably more virulent strain of challenge organism than those in use now would be necessary to overcome the variations in the assay caused by uneven lung uptake of challenge culture. It was also found that the lungs of immunized mice were cleared more rapidly than the lungs of non-immunized mice in the 24-hr. period following the intranasal inoculation.

Introduction

The most widely used method for assaying pertussis vaccine is the N.I.H. intracerebral challenge test in mice (4, 5). The method involves the use of a standard preparation, and is properly designed statistically, but its practical value is limited because of the extremely wide variation displayed by the test animal. The method, when large numbers of mice are used, can apparently distinguish between vaccines of comparatively high or comparatively low potency, but the slope of the dosage response curve is so small that a high degree of precision can be obtained only by using an infinitely large number of test animals.

Other animal tests have been used with even less success. The most promising of the latter methods is the intranasal challenge technique introduced by Burnet and Timmins (1). The use of this technique, in our laboratory, has yielded variable results. Groups of animals immunized intraperitoneally, intranasally, or subcutaneously with a single injection of pertussis vaccine, and challenged intranasally 10 to 12 days later, exhibit extreme variation in survival. This variation is not restricted to immunized animals but has also been noted in normal mice used as controls. At times the normal mice have shown as great or even greater per cent survivals than the immunized animals receiving the same challenge dose. In such cases the assay has no value for it is obvious that the challenge dose, as administered, was not strong enough to measure development of immunity. The challenge dose in assays of this type should be large enough to overcome individual variation in resistance found in normal animals. The failure to do this with

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the intranasal challenge technique could have been due to either one or both of two factors—the low virulence of the organisms or the uneven passage of *H. pertussis* into the lungs of the challenged animals. The present study was undertaken to study the latter factor. During the course of this investigation it was also possible to follow the rate of clearance of *H. pertussis* organisms from the lungs of immunized and normal mice. The results of both studies are reported here.

Materials and Methods

I. Intranasal Challenge Assay Technique

Strains

Kendrick's strain 18-323 (2) was used for the intranasal challenge. The cultures were maintained in the lyophilized state, a fresh tube being opened for each test.

Challenge Cultures

The lyophilized culture was planted on Bordet-Gengou medium containing human blood and proteose peptone, incubated for 72 hr. at 35° C., and then subcultured to the same medium for a further period of 24–36 hr. A second subculture was then made to a similar medium, without proteose peptone. After an incubation of 16 to 18 hr. the culture was scraped carefully from the plate and suspended uniformly in 1 to 2 ml. of 0.85% sterile saline. Prior to using this as the challenge dose any large clumps present were removed by straining the suspension through cotton. Each challenge culture was tested for purity by agglutination and Gram stain.

Standardization of Bacterial Counts

The opacity of the bacterial suspension was adjusted to approximately 10×10^9 organisms per ml. against the World Health Organization Opacity Standard. The intranasal challenge dose was 0.05 ml.—approximately 500 million organisms.

Mice

White mice* weighing initially between 16 and 20 gm. were used throughout the investigation. The individual assays were restricted to animals of the same sex.

Immunization

The vaccines used were diluted serially in 0.85% saline using doubling dilutions, and injected intraperitoneally in 0.5 ml. amounts. Groups of 18 to 22 mice were used for each dilution.

Challenge

The challenge dose was administered 10–12 days after immunization. To facilitate this procedure a 1.0 ml. serological pipette was mounted on a support at a 30° angle. To this was attached a 10 ml. syringe, the plunger of which was controlled by a spring and screw, so that one complete turn of the screw

**Strain bred by Connaught Medical Research Laboratories, University of Toronto.*

caused a delivery of 0.05 ml. The mice, anaesthetized lightly with a mixture containing equal parts of ether and chloroform, were held so that the external nares were adjacent to the tip of the pipette, the inoculum being drawn in during the normal course of breathing. Animals gasping or rejecting the challenge culture were not included in the study. The challenge time was restricted to two hours from the opening of the culture plate to the inoculation of the last mouse. Two control groups of normal mice were used for each challenge. The first group received the challenge at the beginning of this procedure and the second at the conclusion. The animals were observed for 14 days, deaths being recorded daily.

II. Radioisotope Technique

Administration of I^{131}-labelled Vaccine

Pertussis vaccine* labelled with I^{131} by a technique reported previously (3) was administered intranasally, in the manner described above, to two groups of 18 to 20 mice. One was a group of normal animals, the other a group of animals immunized 10 to 12 days previously by intraperitoneal injection with 0.5 ml. of pertussis vaccine. At the same time two identical groups of mice were challenged with radioactive sodium iodide-131†, diluted in 0.85% saline, so that its activity approximated that of the labelled vaccine. To obtain radioactive vaccine of sufficiently high activity it was necessary to increase the concentration of organisms in the 0.05 ml. dose to approximately 6×10^9, i.e., about 12 times the amount in the normal challenge dose. The intranasal challenge dose was administered by the personnel normally doing this work in our routine assays in order to overcome variation due to different personnel. Variation in amounts delivered by the pipette assembly was checked by collecting a number of challenge doses on planchettes and measuring for radioactivity.

Measurement of Radioactivity

Groups of mice were sacrificed at 10 min., 1, 4, 24, and 48 hr. after administration of the labelled material. Blood obtained during exsanguination was collected on planchettes for counting, and fresh weight recorded. The counts obtained were corrected to the weight of a 2.0 ml. sample, which was taken as the approximate mean figure for the total blood volume of a 24 gm. mouse (6), the mean weight of the animals at time of sacrifice. The lungs were removed as soon as possible after death, and connective tissue was removed. The fresh weights of the lungs were recorded and the tissue was retained on planchettes for counting. Other tissues such as liver, kidney, and spleen were spot checked for activity.

Preliminary experimentation indicated that planchette counts did not differ significantly whether the measurements were made on either the intact or homogenized lung. The counts were taken for this reason on intact tissues thereby shortening the time interval between removal of the tissues and

**Supplied by Connaught Medical Research Laboratories, University of Toronto.*
†Supplied by Atomic Energy of Canada Limited, Commercial Products Division, Ottawa.

completion of the radioactivity measurement. This was particularly advantageous where low activities were encountered. The liver, kidney, and spleen samples were measured in the same manner.

A Nuclear Chicago Model DS-1 Scintillation counter and a Model 192 Ultrascaler were used for counting. Corrections for iodine-131 decay were made on all counts.

Results

I. Intranasal Challenge Assay

The type of variation encountered within an assay, and also between repeat assays, may be seen in Table I. The reversal phenomenon, e.g., groups of mice on a low dose of vaccine exhibiting greater survival than mice on a higher dose, will be noted. The wide variation between replicate assays is also evident.

II. Administration of I^{131}-labelled Vaccine

The variation in amounts of labelled vaccine delivered by the pipette assembly used for intranasal challenge administration was assessed by measuring the radioactivity of a number of consecutive doses collected on individual planchettes. The limits of delivery were 0.05 ± 0.0035 ml., a variation of 7%.

The present study involved two separate groups of animals with approximately the same number of immunized and non-immunized mice in each group. The first group received pertussis vaccine labelled with I^{131}; the second group, which was established to indicate the firmness of the attachment of the I^{131} to the vaccine following nasal instillation, received sodium iodide-131.

TABLE I

RESULTS OF ROUTINE ASSAYS OF PERTUSSIS VACCINE USING THE INTRANASAL CHALLENGE TECHNIQUE

		Survival 14 days			
		Assay No. 1		Assay No. 2	
Group	Dilution	Survival	% Survival	Survival	% Survival
Test vaccine	1 : 4	6/16	37.5	14/15	93.3
	1 : 16	1/19	5.3	13/15	86.6
	1 : 64	2/16	12.5	9/18	50.0
	1 : 256	1/17	5.8	9/16	56.2
Reference vaccine	1 : 4	10/16	62.5	12/15	80.0
	1 : 16	5/14	35.7	11/15	73.3
	1 : 64	2/15	13.3	7/15	46.6
	1 : 256	2/15	13.3	9/16	56.2
Control	Before challenge	0/15	0.0	0/15	0.0
	After challenge	0/15	0.0	0/15	0.0

NOTE: *Immunization dose—0.5 ml. intraperitoneally; intranasal challenge dose—500 million organisms; given 10–12 days following immunization.*

TABLE II

PERCENTAGE OF ADMINISTERED SODIUM IODIDE-131 AND OF IODINE-131-LABELLED PERTUSSIS VACCINE PRESENT IN THE LUNGS OF IMMUNIZED AND NON-IMMUNIZED MICE AT VARYING TIME INTERVALS FOLLOWING INTRANASAL INOCULATION

Results are shown as mean percentages ± S.D. ± S.E.M.

Group		Time after intranasal administration				
		10 min.	1 hr.	4 hr.	24 hr.	48 hr.
A	Imm.	56.9 ± 12.2 ± 3.15 (16)*	35.7 ± 12.3 ± 3.3 (15)	22.3 ± 16.1 ± 3.7 (20)	4.64 ± 3.40 ± 0.78 (20)	3.81 ± 1.29 ± 0.30 (20)
	N.I.	52.3 ± 18.2 ± 4.42 (18)	35.5 ± 18.6 ± 4.3 (20)	20.7 ± 16.6 ± 3.8 (20)	9.37 ± 4.98 ± 1.17 (19)	4.37 ± 1.62 ± 0.38 (19)
B	Imm.	14.0 ± 4.98 ± 1.25 (17)	4.20 ± 2.74 ± 0.73 (15)	0.31 ± 0.21 ± 0.048 (20)	0.25 ± 0.10 ± 0.023 (20)	Nil
	N.I.	6.17 ± 3.52 ± 0.83 (19)	5.22 ± 2.24 ± 0.54 (18)	0.63 ± 0.30 ± 0.071 (19)	0.33 ± 0.14 ± 0.033 (19)	Nil

A = *Mice receiving I^{131}-labelled pertussis vaccine.*
B = *Mice receiving sodium iodide-131.*
Imm. = *Immunized animals.*
N.I. = *Non-immunized animals*
**Figures in parentheses denote the number of mice*

TABLE III

PERCENTAGE OF ADMINISTERED SODIUM IODIDE-131 AND OF IODINE-131-LABELLED PERTUSSIS VACCINE PRESENT IN THE BLOOD OF IMMUNIZED AND NON-IMMUNIZED MICE AT VARYING TIME INTERVALS FOLLOWING INTRANASAL INOCULATION

Results are shown as mean percentages ± S.D. ± S.E.M.

Group		Time after intranasal administration				
		10 min.	1 hr.	4 hr.	24 hr.	48 hr.
A	Imm.	0.57 ± 0.50 ± 0.13 (15)*	1.73 ± 0.78 ± 0.21 (15)	2.08 ± 0.89 ± 0.20 (20)	3.03 ± 1.25 ± 0.29 (20)	Nil
	N.I.	0.49 ± 0.50 ± 0.12 (18)	2.33 ± 1.07 ± 0.25 (20)	2.79 ± 1.26 ± 0.29 (20)	3.71 ± 1.88 ± 0.46 (18)	Nil
B	Imm.	14.7 ± 2.26 ± 0.58 (16)	6.35 ± 2.49 ± 0.67 (15)	4.48 ± 2.58 ± 0.59 (20)	2.62 ± 1.20 ± 0.28 (20)	Nil
	N.I.	13.3 ± 3.97 ± 0.94 (19)	9.41 ± 3.18 ± 0.77 (18)	7.45 ± 2.04 ± 0.47 (20)	3.55 ± 2.30 ± 0.54 (19)	Nil

A = *Mice receiving I^{131}-labelled pertussis vaccine.*
B = *Mice receiving sodium iodide-131.*
Imm. = *Immunized animals.*
N.I. = *Non-immunized animals.*
**Figures in parentheses denote the number of mice.*

Preliminary experimentation indicated that sodium iodide-131 alone was adequate for this purpose. Animals receiving non-radioactive iodinated pertussis vaccine plus sodium iodide-131 added just prior to nasal instillation did not show a significantly different uptake of I^{131} when compared to animals receiving the tracer alone.

The results of administering sodium iodide-131 and I^{131}-labelled pertussis vaccine to immunized and non-immunized mice are shown in Table II. Lung samples removed from animals sacrificed at varying times after inoculation were measured for radioactivity. The percentage of the dose present in the lungs at 10 min. following the inoculation was used as the index of the amount reaching the lungs by the intranasal route. The figures taken at later time intervals were used to assess the rate of clearance of tracer from the lungs in the different groups of animals. The level of uptake in the lungs at the 10 min. period was much higher for the group receiving labelled pertussis vaccine than for the group receiving tracer alone. There was no significant difference in the lung uptake between immunized and non-immunized mice receiving labelled pertussis vaccine but there was a significantly higher uptake of tracer among the immunized animals as compared to the non-immunized animals in the sodium iodide-131 control group. The clearance rates from the lungs may also be observed in this table. No activity remained in the lungs of the sodium iodide-131 control group animals at the end of 48 hr. whereas approximately 4% of the administered dose was still present in the lungs of the labelled pertussis vaccine group. Within both the labelled pertussis vaccine and the sodium iodide-131 control groups clearance of radioactivity from the lungs was more rapid in immunized animals. At the end of 24 hr. the activity in the lungs of the non-immunized mice of the labelled pertussis vaccine group was significantly higher than in the lungs of immunized mice. Similarly, in the groups given sodium iodide-131 the four hour level was higher in non-immunized mice.

The levels of radioactivity found in the blood stream are shown in Table III. The 10-min. levels of the tracer in the blood are much higher for the sodium iodide-131 treated animals than for the labelled pertussis vaccine group. At later time intervals, the percentage figures for the control group decreased while those for the labelled vaccine group increased. The blood of non-immunized animals in both groups showed slightly higher radioactivity than the immunized animals between the 1 and 24 hr. period. There was no detectable activity in the blood of either group after 48 hr.

Spot checks of radioactivity at 1, 4, and 24 hr. for both the control and the labelled pertussis vaccine groups revealed the presence of small amounts in the liver, kidney, and spleen. No activity was found at 48 hr. The pattern observed in the results with these tissues was similar to that of the blood, i.e., higher activity for the sodium iodide-131 control group than for the labelled pertussis vaccine group and also higher activity in the organs of non-immunized mice of either group as compared to immunized mice.

Discussion

The present study using radioisotope shows that between animals there is considerable variation in passage and retention of *H. pertussis* organisms in the lungs following intranasal inoculation. Repeated experiments (i.e. the present one and others not reported here) demonstrate, however, that the average lung uptake between groups is reproducible within the limits shown for the standard error of the mean. While the variation, in our opinion, must contribute in part to the wide discrepancies noted in the assay results, it is a factor which could be overcome by use of a strain of *H. pertussis* exhibiting high virulence to mice by the intranasal challenge route. The cultures in use today are obviously not adequate for this purpose.

Seki *et al.* (7), using an intratracheal method of infecting mice with *H. pertussis*, sacrificing the animals immediately after inoculation, and culturing the lungs, found fourfold differences in lung uptake between animals. Our results using I^{131}-labelled vaccine showed variations somewhat less than this, but of the same order. Ten minutes after intranasal inoculation lung uptakes varying from 31.6% to 77.5% were found in immunized animals, and from 25.0% to 83.7% in non-immunized animals. The animal-to-animal variation using the intranasal route is therefore no greater than that found with the intratracheal technique, despite the fact that the latter should be a more direct route of lung infection.

Eleven, or approximately 3%, of the mouse lungs from the total of 333 counted and included in the figures of Table II failed to show a significant count. These animals, some of which were sacrificed at four hours and the remainder at 24 hr. after inoculation did, however, show significant radioactivity in their blood at the time of killing. This may be an indication of the number of animals that did not take the challenge dose properly but swallowed it instead.

Certain differences were found in the lung uptake and clearance of radioactivity between immunized and non-immunized mice. Labelled organisms were cleared more rapidly from the lungs of the immunized animals within the 4 to 24-hr. period than from non-immunized animals. The difference in levels of radioactivity between these groups was highly significant 24 hr. after intranasal inoculation ($P < 0.01$). It seems reasonable to hypothesize that *H. pertussis* antibodies present in the immunized mice were responsible for the more rapid removal of labelled vaccine. On the other hand, we are unable to suggest an explanation for differences observed between immunized and non-immunized mice within the control group where tracer only was administered. In the latter, sodium iodide-131 was retained in higher amount initially in the lungs of immunized but here again clearance was more rapid in these animals. The blood levels in both groups of mice showed a higher radioactivity in the non-immunized animals than in the immunized ones from 1 to 24 hr. after intranasal administration. This is probably due to a slower rate of clearance of tracer from the bodies of the non-immunized mice.

It is difficult to assess how much of the variation in the assays was due to differences in lung uptake and how much to the low virulence of the *H. pertussis* challenge organism. It seems clear, however, that this method of assay will remain unsatisfactory unless a challenge culture is introduced, with enough virulence to overcome or at least minimize the differences found in resistance between normal mice, and in addition to overcome the difference noted in lung uptake between animals.

Acknowledgments

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PHYSIOLOGICAL BASES OF MORPHOGENESIS IN FUNGI

5. EFFECT OF SELENITE AND TELLURITE ON CELLULAR DIVISION OF YEASTLIKE FUNGI[1]

By W. J. Nickerson, W. A. Taber,[2] and G. Falcone[3]

Abstract

Concentrations of selenite and tellurite that are somewhat inhibitory to the growth of yeastlike fungi are shown to cause a variety of filament-producing organisms to grow exclusively in a yeast-phase condition. This effect is shown not to be a selective inhibition of the filamentous type of growth, but a promotion of cellular division. With subbacteriostatic concentrations of many antimicrobial agents it is well known that a filamentous type of microbial growth can be obtained owing to selective inhibition of cellular division, but this is believed to be the first instance in which antimicrobial agents have been shown to enhance division of normally filamentous cells. Analogies are drawn between the properties of sulphur-containing compounds and those with selenium or tellurium. Information is presented on the occurrence of a sulphur-rich protein in the cell wall of yeasts and on the occurrence of organically bound selenium in the cell wall of organisms grown on selenite.

Introduction

The toxic effects of salts of selenium on microorganisms and on animals are well known (1, 14, 16); similarly, the conversion of selenite to dimethyl selenide by certain fungi has been extensively studied (2). Less well known, however, are instances wherein selenium apparently replaces its analogue, sulphur, and enters into the formation of compounds which participate in the metabolism of the organism. There are, for example, seleniferous plants that may accumulate several thousand parts per million of selenium, which is considered by Trelease (16, 17) to be essential for the growth of certain of these plants. In a study of the enzymatic reduction of sulphite, selenite, and tellurite by yeasts (9, 10), it was observed that $SeO_3^=$ and $TeO_3^=$, but not $SO_3^=$, promoted cellular division in filamentous strains of *Candida albicans*, whereas all three substances were accumulated and actively reduced.

This paper describes effects of selenite and of tellurite on growth (defined as an irreversible increase in volume) and cellular division processes in a variety of yeastlike fungi. The isolation of a mutant strain of *C. albicans* highly resistant to selenite is reported.

Materials and Methods

Cultures

Descriptions of the microbiological techniques and strains of yeasts employed have been given previously (11). The medium designated by GGY contained in grams per liter of distilled water: glucose, 10.0; glycine, 10.0; and yeast

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[2]*Prairie Regional Laboratory, Saskatoon, Saskatchewan.*
[3]*Waksman-Farmitalia Postdoctoral Fellow. Present address: Istituto di Pathologia Generale dell' Universita di Napoli, Naples, Italy.*

extract, 1.0. The principal test organisms were *Candida albicans* strain 582 (ATCC No. 10261) and a filamentous mutant derived therefrom, strain 806 (ATCC No. 10259). Other organisms used included strains of black yeasts: *Pullularia pullulans*, kindly provided by Professor C. M. Haenseler of the Department of Plant Pathology, Rutgers University; and *Hormiscium dermatitidis*, obtained through the courtesy of Dr. Margarita Silva, Department of Medical Mycology, College of Physicians and Surgeons, Columbia University. Strains of *Geotrichum* were isolated in this laboratory.

Reagents

Selenite was employed as the sodium salt, obtained from the Amend Drug Company, New York; potassium tellurite was obtained from Eimer and Amend, New York. Concentrated aqueous solutions of these salts were sterilized in an autoclave (20 min. at 120° C.), and then suitably diluted in sterile water blanks, after which appropriate aliquots were added aseptically to sterile culture medium immediately prior to inoculation.

Viable Cell Counts

For the count of viable cells in cultures grown in different levels of selenite, an aliquot was taken from each flask (agitated thoroughly to insure a homogeneous suspension) and diluted serially in geometrical progression of 1:10 in selenite-free GGY broth. From appropriate dilutions, aliquots of 0.5 ml. were taken in quadruplicate and added to tubes containing 3 ml. of molten agar medium (0.6% agar in GGY broth) maintained at 48° C. in a water bath. After thorough mixing of the contents, each tube was poured and distributed evenly over a layer of solidified GGY agar medium contained in a 10 cm. Petri plate. The count of colonies developed on each plate was made after 49 hr. incubation at 28° C.

Effect of Selenite on Form Development of C. albicans

After about five days' incubation on GGY agar medium, normal strains of *Candida albicans* characteristically make a filamentous growth from the free borders of streak cultures. If selenite is incorporated into the medium, the development of filaments is suppressed to an increasing degree with increasing concentration of selenite; there is no comparable inhibition of the yeast-type of growth in these selenite-containing agar media. These two points are illustrated in Figs. 3–6. The permission of yeast-like growth (growth by free-cell formation) and suppression of filament formation by selenite and tellurite may be due to selective inhibition of filamentous growth—i.e. inhibition of growth that occurs in the absence of cellular division. Alternatively, the apparent promotion of cellular division may result from an effect of selenite that prevents the uncoupling of cellular division from growth, an uncoupling that is the underlying basis of filament formation in aging cultures (8, 10). The experiments which are now to be reported will show that the second of these alternatives is to be favored.

TABLE I

EFFECT OF SELENITE CONCENTRATION ON GROWTH OF YEASTS IN CONTINUOUSLY AGITATED CULTURES AT 28° C.

Organism and concentration of Na_2SeO_3 in GGY medium	Growth (optical density)					
	24 hr.		48 hr.		112 hr.	
	Growth	% inhibition	Growth	% inhibition	Growth	% inhibition
C. albicans 582						
Zero	0.132	—	1.094	—	1.290	—
10^{-5} *M*	0.070	47.0	0.910	16.8	1.130	12.4
10^{-4} *M*	0.000	100.0	0.268	75.5	0.466	63.9
C. albicans 806						
Zero	0.232	—	0.930	—	1.070	—
10^{-5} *M*	0.098	57.7	0.610	34.4	1.040	2.8
10^{-4} *M*	0.000	100.0	0.000	100.0	0.370	65.4
Geotrichum 105						
Zero	0.700	—	0.996	—	0.966	—
10^{-5} *M*	0.420	40.0	0.900	9.6	1.020	0.0
10^{-4} *M*	0.072	89.7	—	—	0.326	66.3

Data in Table I and in Fig. 1 show the effect of increasing concentrations of selenite on the crop yield and rate of growth of both the yeast form (strain 582) and the filamentous form (strain 806) of *C. albicans*. It is clear that the growth of both forms is about equally sensitive to selenite. Furthermore, the near identity in the rates of synthesis of protoplasmic mass in the normal strain (582) and in the filamentous strain (806) of *C. albicans* (Table I) provide a clear indication that the form the organism takes is not determined in the first instance by the growth process. The operation of a cellular division process appears to be superimposed on the growth process, and may operate (or not) without impairment to the rate of growth. This situation has also been shown conclusively for normal and filamentous growth with *Bacillus cereus* (13).

A filamentous mutant of *C. albicans* (strain 806) has been studied in some detail and the locus of genetic block in this divisionless mutant has been identified as a dissociable metallo-flavoprotein that participates in the transfer of hydrogen in a reaction which is essential to division, but not to growth (12). This normally filamentous strain grows moderately well, largely in a single-celled, budding yeast form, when exposed on agar to 10^{-4} *M* selenite. Partial inhibition of growth occurs at higher concentrations of selenite. This is not a selective suppression of growth of the filamentous form but a reversal of the distribution of filamentous, transition, and yeast types on the addition of selenite to the growth medium (Figs. 7–8 and Table II).

The presence of selenite does not cause a selection of back mutants to the yeast type. On transfer to a medium devoid of selenite, all of the cells revert to a filamentous character (Table II). Selenite functions in some manner to permit cellular division to occur in the genetically blocked, divisionless strain.

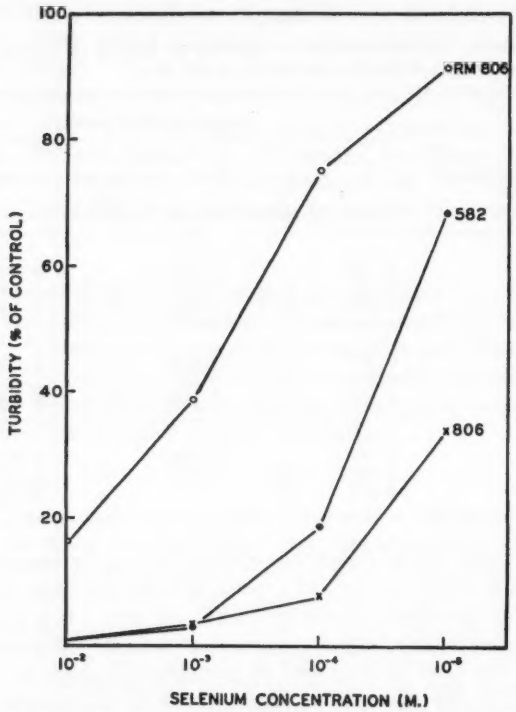

FIG. 1. Effect of selenite concentration on growth of three strains of *C. albicans*. Cultures grown in GGY liquid medium with continuous agitation for 24 hr. Data expressed as per cent of growth in control cultures without selenite. Strain 582 is a normal strain of *C. albicans*, 806 a filamentous strain, and RM 806 is a selenite-resistant mutant derived from 806. Similar sensitivities of the yeast (582) and filamentous (806) growths are apparent, as is the pronounced resistance of RM 806.

TABLE II

EFFECT OF SELENITE ON THE MORPHOLOGY OF *C. albicans* STRAIN 806

Concentration of Na_2SeO_3 in GGY medium	Morphology		
	% yeast cells	% transition forms	% filamentous cells
Zero	5	5	90
10^{-5} *M*	50	13	37
10^{-4} *M*	78	10	12

Promotion of Cellular Division in Other Yeastlike Fungi

A very striking example of the effect of selenite on the promotion of cellular division is found with *Hormiscium dermatitidis*. This black yeastlike fungus is almost completely filamentous in appearance on the GGY medium. In the

presence of 10^{-3} *M* selenite growth is partially inhibited, but that which occurs is almost entirely in the yeast form. No such effect was observed with strains of *Cladosporium*, which is a true filamentous fungus in which the capability of developing a budding form has not been reported.

In *Pullularia pullulans* (strain 301) on GGY agar the cells of the inoculum develop as budding yeasts with occasional, limited hyphal formation. The growth is colorless for about the first 48 hr., but by 120 hr. is greenish black.

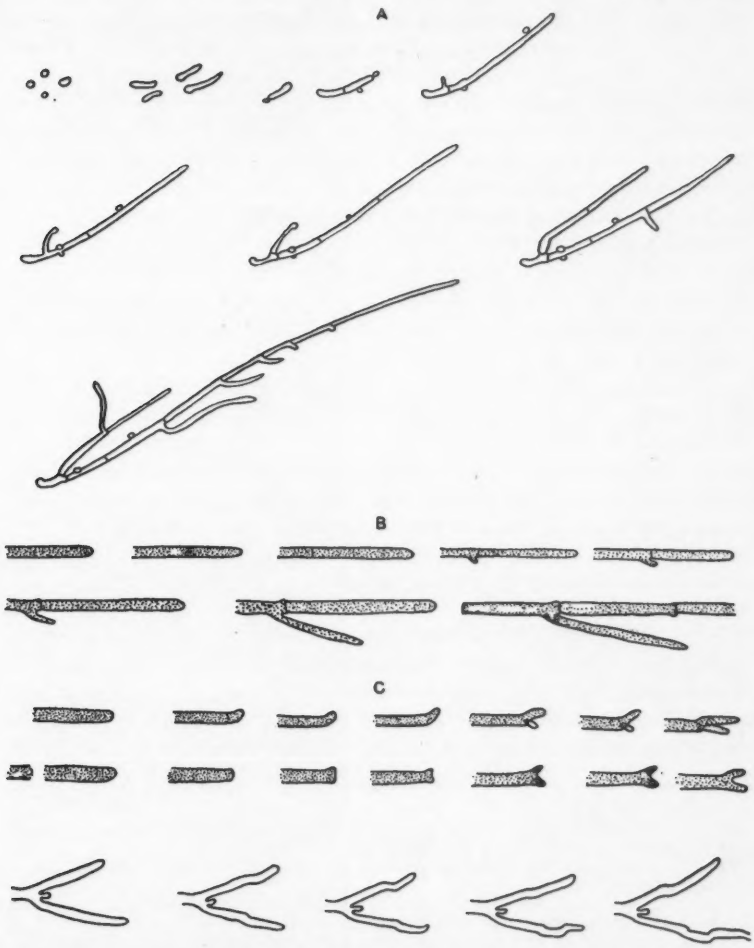

FIG. 2(A). Growth habit of *Geotrichum* sp. on GGY agar, during first 16 hr. incubation at 25° C. Germination of arthrospores (upper left) gives rise to filaments which grow by apical elongation; lateral branching follows septation of hyphae as shown in (B). In (C) is shown the dichotomous and near dichotomous branching which occurs at the periphery of colonies more than 24 hr. old.

After about 48 hr. hyphal development occurs abundantly at the periphery of colonies. These filaments become pigmented and develop blastospores singly and in clusters.

With 10^{-3} *M* selenite the normal black pigmentation and filamentation of *Pullularia* are completely suppressed and the growth, which is fairly heavy, remains in the budding yeast form. After about one week the growth is pink to light red, owing to the accumulation of selenite and reduction thereof to selenium.

The germination of arthrospores of *Geotrichum* sp. (strain 105) inoculated onto GGY agar gives rise to an abundant development of filaments which grow by apical elongation as shown in Fig. 2A. Aerial hyphae develop abundantly and fragment rapidly to form dense masses of arthrospores which impart a "yeast" appearance to the streak of growth (Fig. 9). From the periphery of colonial and streak growths there develops, after about 24 hours' incubation, a dense prostrate mycelium that may extend several centimeters from the margin (Fig. 9). This mycelial development is composed of hyphae which exhibit dichotomous branching (Figs. 2B and 11).

With 10^{-3} *M* selenite present in the GGY medium the arthrospores of *Geotrichum* in the inoculum multiply by elongation and septation; hyphal development as shown in Fig. 2A does not occur and the microscopic appearance of the growth after 24 hr. is shown in Fig. 12. In a macroscopic view of a plate with selenite (Fig. 10), complete absence of peripheral mycelial development is noted. The yeast growth is pink owing to the accumulation of selenite and reduction to selenium.

On a medium without selenite both *Geotrichum* and *Pullularia* exhibit a marked tendency to grow as filaments. Methods for maintaining a balance between growth and division in these organisms, through nutritional control, have not yet been worked out, as has been done for *Candida* (12). However, in these forms, as well as in *C. albicans*, selenite acts to promote cellular division.

Isolation of Selenite-resistant Mutant of C. albicans

In cultures of the filamentous mutant, strain 806, of *C. albicans* on GGY agar containing high concentrations of sodium selenite, there developed some small dark red colonies. The strongly reducing cells of these colonies were exclusively of the yeast type. A sample was taken from one of these colonies,

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FIG. 3. *Candida albicans*, normal strain 582, incubated for seven days on GGY agar. Note filamentous growth arising from free borders of streaks.

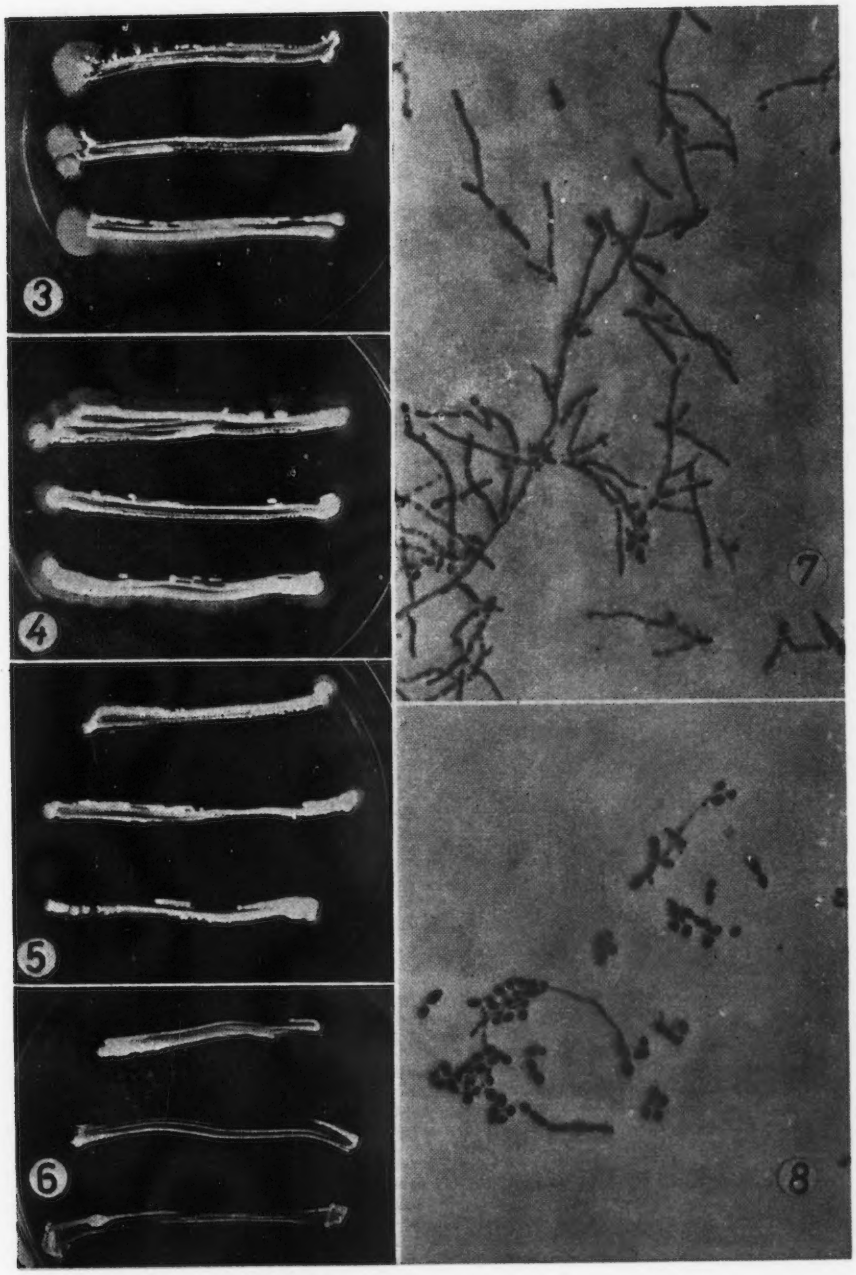
FIG. 4. Same, plus 10^{-5} *M* sodium selenite; no appreciable effect on yeast or filamentous development.

FIG. 5. Same, plus 10^{-4} *M* sodium selenite; considerable repression of filamentous development but no appreciable inhibition of yeast growth.

FIG. 6. Same, plus 10^{-3} *M* sodium selenite; complete suppression of filamentous development with only partial inhibition of yeast growth (which is salmon-colored as a result of accumulation of metallic selenium from reduction of selenite).

FIG. 7. Phase contrast photomicrograph of filamentous strain 806 of *C. albicans* grown for 24 hr. in GGY medium with continuous aeration.

FIG. 8. Same as for Fig. 7 but cells grown in medium with 10^{-3} *M* selenite.



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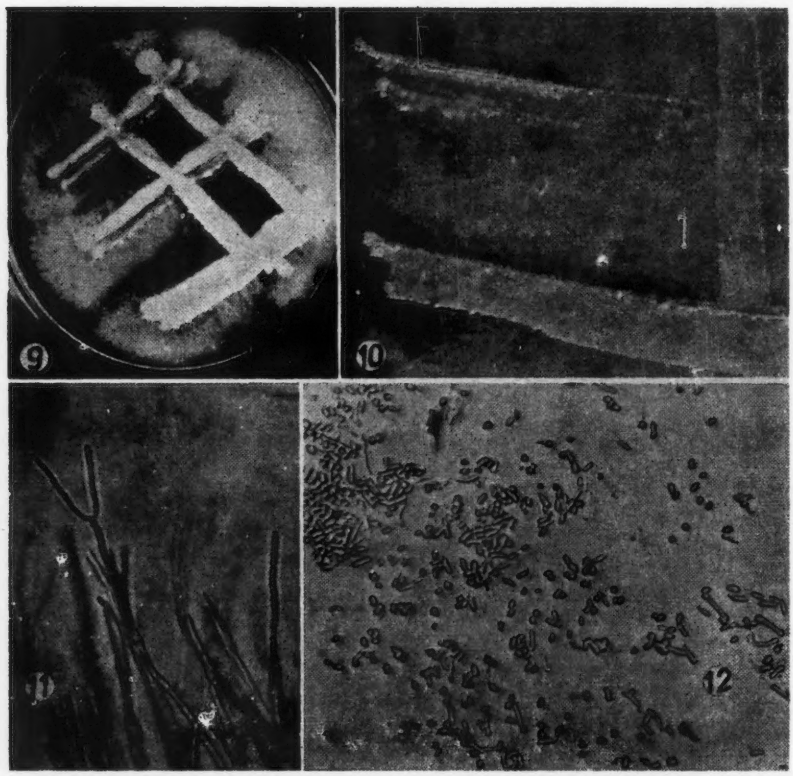


FIG. 9. Culture of *Geotrichum* strain 105 grown for seven days on GGY agar. Extensive mycelial development extends to rim of Petri dish.

FIG. 10. Same as for Fig. 9 but on medium containing 10^{-3} *M* selenite.

FIG. 11. As for Fig. 9, photomicrograph of *in situ* growth at periphery of mycelial growth. Note dichotomous branching of filament.

FIG. 12. As for Fig. 10, photomicrograph of arthrospore masses obtained on selenite, and comprising streak growths shown in Fig. 10.

suspended in broth, diluted serially, and plated onto GGY agar containing sodium selenite at a final concentration of 10^{-2} *M*. From the colonies that developed on this plate, one of the type described was selected and again suspended in broth, diluted serially, and plated on agar containing 10^{-2} *M* Na_2SeO_3. This operation was repeated twice more to obtain the highly resistant strain designated as RM 806.

This strain is able to grow rapidly in the presence of high concentrations of selenite; the levels of growth attained after 24 hr. incubation in continuously agitated cultures with different concentrations of selenite are shown in Fig. 1. In comparison with strains 582 and 806, the resistant nature of RM 806 is readily apparent. Although strain RM 806 was picked on the basis of its marked selenite-reducing ability, its ability to grow on high concentrations of selenite does not appear to be related directly to its ability to reduce selenite. In liquid media containing 10^{-3} *M* Na_2SeO_3, multiplication begins very shortly after inoculation (even when the inoculum has been grown on a medium without selenite) and a dense turbidity is achieved by 18–24 hr. Noticeable reduction of selenite to red, metallic selenium[4] occurs in such cultures only after about 48 hr. incubation. In suspensions of non-multiplying (resting) cells that have been grown on a medium free of selenite, the addition of selenite results in very marked production of reduced selenium within 12 hr. incubation. Biochemical studies on the metabolism and composition of strain RM 806, and on the mechanism of selenite reduction, are in progress and will be reported subsequently.

The macroscopic appearance of streak cultures of RM 806 on agar medium at different levels of selenite is shown in Figs. 13–18. Unlike the filamentous strain 806 from which it was derived, RM 806 grows almost completely as a

TABLE III

RETENTION OF SELENITE RESISTANCE IN MUTANT STRAIN RM 806 OF *C. albicans* IN THE ABSENCE OF SELENITE

Addition to GGY medium	Cells per ml. of 48 hr. broth culture	
	Inoculum grown in GGY broth (7 passages in GGY broth)	Inoculum grown in GGY broth containing 10^{-3} *M* Na_2SeO_3
None	8.1×10^6	7.3×10^5
10^{-5} *M* Na_2SeO_3	13.0×10^6	7.2×10^5
10^{-4} *M* Na_2SeO_3	10.5×10^6	6.8×10^5
10^{-3} *M* Na_2SeO_3	10.6×10^6	6.4×10^5

[4]*The red intracellular material is usually assumed to be the red form of metallic selenium. This has been proved to be the case by breaking the cells according to the method of Lamanna and Mallette (5) and removing the red particulate matter by differential centrifugation and washing. This fraction was insoluble in water, but soluble in carbon disulphide and in* H_2SO_4. *From carbon disulphide it could be precipitated by the addition of water; such behavior is characteristic of the red amorphous form of selenium. Other characteristic tests for selenium, including the codeine phosphate test, were positive. Organically bound selenium also occurs in C. albicans grown on selenite. Studies on this material will be reported subsequently.*

budding yeast, even in the absence of selenite. It has the property, however, of forming filaments from the free borders of streak growths on continued incubation in the absence of selenite (Fig. 13). As with normal strains of *C. albicans*, filamentation is suppressed on the addition of selenite (Figs. 14–17) and the resultant heavy growth is exclusively in the yeast form. The very intense reduction of selenite in this strain is readily apparent from Figs. 16–18.

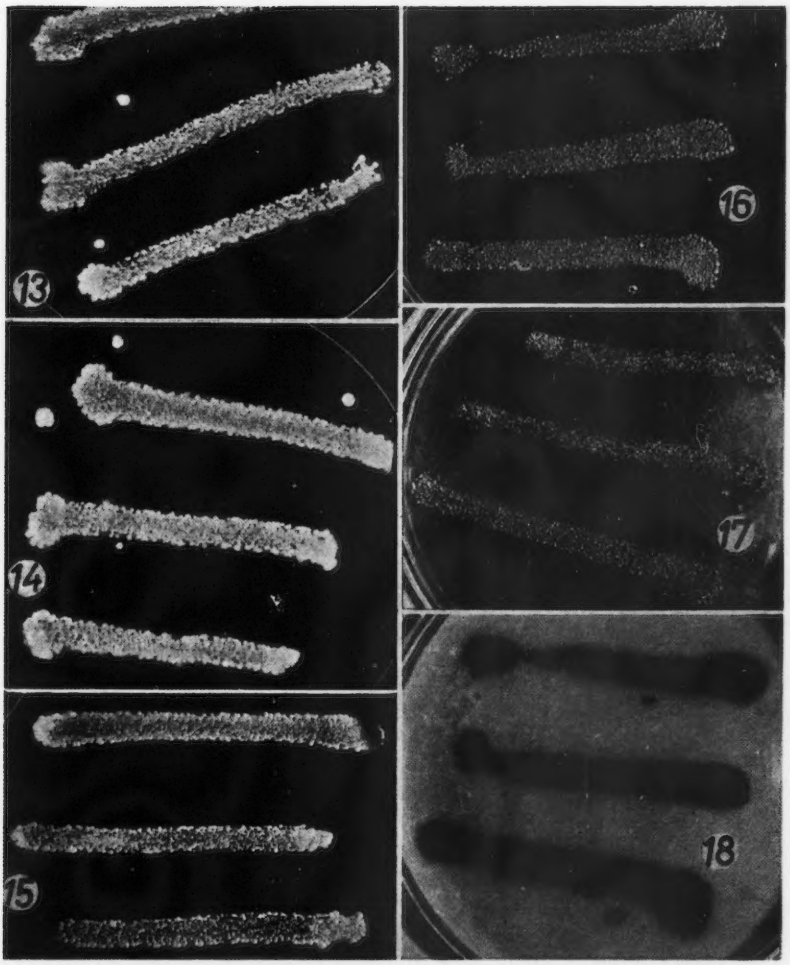
Table III shows the retention of selenite resistance in RM 806 after repeated passage in medium without selenite. There is no loss in selenite resistance in this strain after repeated passage in the absence of selenite. These data also show that after 48 hr. incubation the levels of growth of RM 806 with 10^{-3} *M* selenite and without selenite are approximately the same (the values in Fig. 1 are for 24 hr. incubation).

Bearing of Cellular Morphology on Pathogenicity of C. albicans

Mackinnon (6) reported strain 582 of *C. albicans* to be highly pathogenic for experimental animals, whereas the filamentous mutant strain 806 was non-pathogenic. In tests conducted in collaboration with Dr. Philip C. Eisman, J. V. Tanzola, and S. G. Geftic of the Department of Bacteriology, CIBA Pharmaceutical Products, Inc., Summit, N.J., the findings of Mackinnon have been confirmed with mice as the experimental animal. Curiously, strain RM 806, which is a mutant derived from the non-pathogenic strain 806, is virulent for mice. On intravenous injection test strain 325 of *C. albicans*, for example, killed 14 out of 14 mice within nine days, with an average survival time of 4.7 days. Strain RM 806 killed 15 out of 15 mice within 20 days, with an average survival time of 13.5 days. Eisman, Geftic, and Mayer (4) have also found filamentous strains of *C. albicans* to be less virulent than yeast-type strains. The findings with strain RM 806 are of considerable interest since the regain of pathogenicity is associated with a return of the strain to the yeast type of growth. These studies will be reported in detail elsewhere.

Effect of Tellurite on C. albicans

Only preliminary experiments have been conducted with tellurite, but these are sufficient to indicate that the action of this material is analogous to that of the closely related selenite. Addition of increasing concentrations of potassium tellurite to GGY agar resulted in some inhibition of growth of *C. albicans* strain 806 (to about the same extent as with selenite), but the fairly abundant growth that developed after seven days' incubation at 10^{-4} *M* and 10^{-3} *M* K_2TeO_3 was intensely black (because, quite probably, of the production of black metallic tellurium as an intracellular reduction product) and was almost completely in the yeast form. In continuously agitated liquid cultures tellurite was reduced more rapidly than selenite; in fact, after 24 hr. incubation, blackening due to tellurite reduction was so intense as to prevent turbimetric or gravimetric determination of growth.



FIGS. 13–18. Growth of *C. albicans* strain RM 806 on GGY agar after seven days' incubation at different levels of selenite. In Fig. 15, no selenite; tufts of filamentous growth apparent from free margins of streaks. In Fig. 14, 10^{-5} *M* selenite; Fig. 15, 10^{-4} *M* selenite; Fig. 16 and 18, 10^{-3} *M* selenite; Fig. 17, 10^{-2} *M* selenite. Suppression of filamentation and increased selenium content are readily observed with increasing concentration of selenite. Fig. 18 is the same as Fig. 16, but with a white background to show the intense color developed at 10^{-2} *M* Na_2SeO_3 from selenite reduction.

Discussion

So far as the authors can ascertain, the selective action of selenite (and tellurite) in promoting division (i.e. favoring free-cell formation) is a unique effect for a substance that is a growth-inhibitor for so many forms of life. It is well known that very low concentrations of many growth-inhibitors, including many antibiotic substances, cause a selective inhibition of division, but selective promotion of division has heretofore been encountered only among sulphhydryl (—SH) containing substances—this being the original basis for the assumption that some form of —SH substance plays a major role in cellular division.

Similarities in the chemical and physical properties of compounds containing sulphur, selenium, and tellurium (the "sulphur family") have been recognized for many years. Similarities in many of the physiological properties of selenium and tellurium analogues of sulphur-containing compounds have been appreciated more recently. Salts of selenium appear to inhibit competitively the enzymatic reduction of sulphite and sulphate (9, 18), and to compete with sulphur for methyl donors in microorganisms (2, 18, 19). Considerable selenium and tellurium may be bound to protein in higher plants and in microorganisms (3, 14, 16, 18); the exact nature of the binding is not certain, but there is some evidence to show that selenium-containing amino acids may be incorporated into proteins (14).

Sulphur contained in protein occurs to a large extent in an oxidized state in disulphide (—S—S—) linkages; the sulphhydryl group (—SH), which is essential for the activity of many enzymes, is readily subject to oxidation both in the cell and in isolated proteins. It is well known that reduction of some of the disulphide bonds of keratin, and subsequent cross-linking of the exposed —SH by oxidation, are the essential steps in hair-waving (15). To phrase this latter example in general terms, a protein in a partially reduced condition can undergo plastic deformation, whereas complete or nearly complete oxidation to the —S—S— condition constitutes, quite literally, a vulcanized state. In contrast to the lability of —SH in protein, organically bound (protein bound) selenium has been reported to occur in the reduced state (—SeH) and to be oxidized with difficulty.

Recently we have separated from isolated cell walls of baker's yeast a protein that contains 2.1% sulphur. This protein is firmly bound to the mannan component of the wall; the mannan–protein complex forms a considerable portion of the cell wall. In preparations of isolated cell walls from the RM 806 strain of *C. albicans* grown on 10^{-2} *M* selenite, the presence of an appreciable amount of organically bound selenium has been determined. Evidence has been mounting over the years that a reduced (—SH) condition in functional proteins is a necessary condition for mitosis (7) and for cellular division (7, 8, 10). Studies are now in progress that aim at elucidating the role that the yeast cell wall mannan–protein may play in cellular division, and to explore the possibility that a cell wall protein containing —SeH might fill the role of one containing —SH.

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THE METABOLIC STABILITY OF NUCLEIC ACIDS IN ESCHERICHIA COLI[1]

By L. Siminovitch and A. F. Graham

Abstract

P^{32} incorporated in ribo- and desoxyribo-nucleic acids during multiplication of *Escherichia coli* was retained there as nucleotide phosphorus during subsequent generations. There was little or no unequal exchange of P^{32} between the nucleotides of either nucleic acid during bacterial multiplication. The kinetics of assimilation of inorganic P^{32} from medium of constant specific activity into the nucleotides of *E. coli* nucleic acids indicated that phosphorus was incorporated faster into RNA than DNA. *E. coli* was propagated for two minutes in the presence of inorganic P^{32} and the rate at which this "two minute" P^{32} was incorporated into RNA and DNA phosphorus was measured. About 50 to 60 min. was required for both RNA and DNA phosphorus precursor pools to empty. P^{32} was incorporated into RNA at a faster rate than into DNA. The final ratio $RNAP^{32}/DNAP^{32}$ was 50% higher than would be expected from the ratio RNAP/DNAP in *E. coli*. An equal exchange of phosphorus between RNA and DNA does not occur during bacterial multiplication.

Introduction

The early investigations of Schoenheimer and his colleagues showed clearly that many of the constituents of animal tissues are in a state of dynamic equilibrium (12). This concept of continuous degradation and resynthesis, or turnover, of such tissue components as proteins and nucleic acids has been extended by later investigators to include the notion that there is an *intracellular* turnover of these constituents. Recently, as a result of work on bacterial systems, the validity of this extension has been challenged (3). Hogness, Cohn, and Monod (3) and Koch and Levy (5) have shown, in fact, that the proteins in *Escherichia coli* are metabolically stable during the logarithmic phase of growth. Also, Hershey (2) has found that P^{32}, incorporated into the ribo- and desoxyribonucleic acid (RNA and DNA) fractions of *E. coli*, is conserved through several generations of bacterial multiplication. He showed as well that an unequal exchange of P^{32} between RNA and DNA probably does not occur, and that there is no replacement of nucleic acid bases.

In the present work we have found that P^{32} once incorporated into the nucleic acids of *E. coli* is retained there as nucleotide phosphorus, and, moreover, that little or no unequal exchange of P^{32} between nucleotides of either nucleic acid takes place. In addition, our results show that an equal exchange of P^{32} between RNA and DNA is highly unlikely.

Materials and Methods

Growth of Cultures

Escherichia coli B was grown in a synthetic medium containing in grams per liter of distilled water: $CaCl_2$, 0.01; $MgSO_4.7H_2O$, 0.12; NH_4Cl, 0.44;

[1]*Manuscript received June 18, 1956.*
Contribution from the Connaught Medical Research Laboratories, University of Toronto, Toronto, Ontario. Supported in part by grants from the National Cancer Institute of Canada; The National Institutes of Health, United States Public Health Service; and the W. B. Boyd Memorial Fund.

NaCl, 0.59; KCl, 0.15; $FeCl_3$, 0.003; tris (hydroxymethyl)aminomethane buffer, 12.1; glucose, 4.0. Phosphate was sterilized separately and added to this medium as Na_2HPO_4 in varying amounts depending on the experiment. Glucose was sterilized separately as a 4% solution and added to the medium just prior to use. The final pH of the medium was about 7.4. Stock cultures were begun from a slant every two weeks and maintained in the synthetic medium by daily transfer.

Bacterial concentrations were measured turbidimetrically at 425 mμ in a Fisher Electrophotometer standardized against the actual number of bacteria by plate count; 10 optical density units were equivalent to 1.5×10^8 bacteria per ml. Cultures were grown in two ways. For small volumes a special type of flask[2] was used which permitted rapid measurement of the optical density without the need for sampling; the culture was shaken vigorously at 37° C. For larger volumes the cultures were placed in bottles of up to 10 liters capacity and these bottles were rotated on their long axes at 400 r.p.m. at 37° C. All experiments were done with bacteria in the logarithmic phase of growth. The generation time was 54 to 56 min.

Separation of Bacterial RNA and DNA Nucleotides

For analysis, samples were quickly chilled to about 5° C. and trichloroacetic acid (TCA) was added to a final concentration of 5%. After 15 min. the suspension was centrifuged at 5000 *g*. The sediment was washed once with 30 ml. of cold 5% TCA and once with 30 ml. of cold absolute ethanol. It was then suspended in 30 ml. of ethanol and boiled for 15 min. After centrifugation, the boiling alcohol procedure was repeated on the residue. Six milliliters of 0.3 *N* potassium hydroxide was then added to the final residue and this suspension allowed to stand at 37° C. for 18 hr. The digest was adjusted to pH 7.0, centrifuged, and the small residue discarded. The supernatant was cooled to 4° C., acidified to about pH 6 with hydrochloric acid, and cold TCA added to a concentration of 5%. After centrifugation, the supernatant, containing the RNA nucleotides, was removed. The precipitate was taken up in cold 0.1 *N* hydrochloric acid and reprecipitated with 5% TCA. This precipitate contained the polymerized DNA (11).

The solution of RNA nucleotides was brought to pH 4 with potassium hydroxide and then passed through a bed of Merck activated charcoal 0.5 cm. $\times$ 0.6 cm.^{2}, supported on a coarse sintered glass disk. Such a column adsorbs mononucleotides and allows many of the salts and other impurities to pass through. The column was washed with 5 to 10 ml. of distilled water, and then 10 ml. of a solution of 48% water, 50% absolute ethanol, and 2% concentrated ammonium hydroxide (S.G. 0.880) was added.[3] This eluted the mononucleotides, which were then evaporated to dryness on a water bath at 50° C. in a stream of nitrogen.

[2]*Similar in design to the Nepheloflask manufactured by Bellco Glass Co., Vineland, New Jersey.*
[3]*We are indebted to Dr. C. W. Helleiner, Biochemistry Department, University of Toronto, for describing the details of this technique to us before publication.*

The DNA sediment was taken up in water, adjusted to pH 7, magnesium sulphate (to 0.03 *M*) and desoxyribonuclease were added, and the solution was incubated for 12 to 18 hr. at 37° C. The pH of the solution was then adjusted to 9.3 and snake venom phosphodiesterase (4) added. Following incubation at 37° C. for 12 to 18 hr. the solution was cooled and TCA added to give a final concentration of 5%. This precipitated the enzyme proteins and any undigested material. After centrifugation the supernatant was adjusted to pH 4.0 and passed through a charcoal column as with the RNA fraction. About 50% of the DNA phosphorus was converted to TCA soluble form by the enzymes employed here.

Subsequent purification of the mononucleotides from RNA and DNA involved three steps. The sample was first subjected to paper ionophoresis at pH 3.5 in 0.05 *M* ammonium formate buffer (8). With small amounts of material this procedure separated the four mononucleotides from each other. However, in practice, with large amounts, it was found that clear-cut separation of two bands only was obtained: adenylic and cytidylic acids in one and guanylic and uridylic acids (or thymidylic acid) in the other. These two bands were eluted separately from the paper by displacement with water and the solutions evaporated almost to dryness. Each concentrate was placed in one corner of a 9.5 cm. × 18 cm. sheet of Whatman No. 3 filter paper and run downwards in the solvent mixture described by Magasanik *et al.* (7). The solvent consisted of isobutyric acid, 163 ml.; water, 96.7 ml.; and concentrated ammonia, 3.3 ml. This solvent separated adenylic acid from cytidylic acid and, to some extent, guanylic acid from thymidylic acid, but not guanylic acid from uridylic acid. The paper was dried and strips containing the various nucleotides were cut out perpendicular to the direction of solvent flow. Each strip was subjected to a descending run in a solvent containing 95% ethanol, 75 ml.; 1 *M* ammonium acetate (pH 7.5), 30 ml. (9). In this way guanylic acid was separated from either uridylic or thymidylic acids.

The isolated nucleotides were then eluted with water and their extinctions in 0.1 *N* hydrochloric acid measured in a Beckman spectrophotometer at wave lengths of maximum absorption. The phosphorus content of each nucleotide was calculated from the molecular extinction coefficients given by DeLuca *et al.* (1) for ribonucleotides and Lesley and Graham (6) for desoxyribonucleotides. Radioactive phosphorus was estimated in a liquid counter (15) and specific activities of the nucleotides were calculated as counts per min. per μgm. of phosphorus.

Results

Stability of Bacterial Nucleic Acid Phosphorus

In general, bacteria were labelled uniformly with P^{32}, allowed to multiply through several generations in P^{32} free medium, and at intervals the radioactivities of bacterial RNA and DNA nucleotides determined. If turnover of nucleic acid phosphorus takes place, one or more of the nucleotides should have its P^{32} replaced by P^{31} from the medium and its radioactivity should diminish.

A 10 ml. culture of bacteria, containing, in the medium, 15 μgm. of inorganic phosphorus per ml. and 1 mc. of carrier-free inorganic P^{32}, was allowed to multiply through five generations to a final density of 10^9 cells per ml. The bacteria were centrifuged, washed twice in isotope free medium, and suspended in 100 ml. of P^{32}-free medium containing 1500 μgm. of inorganic phosphorus per ml. Uptake of P^{32} not removed by the washing procedure was reduced to a negligible amount in this phase of the experiment by the high concentration of unlabelled phosphate. During subsequent multiplication of the labelled bacteria, after 0, 2.5, and 5 generations, equal aliquots of the culture were treated in one of the following ways. (1) In two experiments equal volumes of a suspension of unlabelled carrier bacteria were added to the experimental samples. The carrier suspension added to each sample contained about 10^{12} bacteria. Each sample was then treated as described above for the separation of RNA and DNA mononucleotides. (2) In another experiment a large amount of unlabelled carrier bacteria was subjected beforehand to several steps in the nucleic acid separation procedure. After the alkaline hydrolysis stage (0.3 *N* potassium hydroxide) the resulting solution was stored in the frozen state. Samples from the experimental culture were treated separately to the same point and then equal volumes of alkaline carrier solution were added to each. Separation of nucleic acids and mononucleotides was then effected as before. The second method of adding carrier bacteria was employed in later experiments since it proved more convenient. With both methods the specific activities of the separated mononucleotides were determined. Table I shows the results obtained in three experiments after the data had been normalized as explained in the footnote to the table.

With the technique employed here the number of bacteria per sample of experimental culture is insignificant in comparison to the number of carrier bacteria. The specific activities of the separated nucleotides are determined essentially, then, by the amount of nucleic acid P^{32} in the experimental culture and the amount of nucleic acid phosphorus added in the carrier. Since equal volumes of the culture were withdrawn during the course of bacterial multiplication, and since an equal amount of carrier bacteria was added to each sample, the measured specific activities of the separated nucleotides are comparable to each other. Moreover, since the carrier was in large excess, such specific activities essentially represent, although do not equal, the amount of P^{32} contained in the respective nucleotides per unit volume of the experimental culture before dilution with carrier. Unequal material losses may be incurred during the analytical procedures without the validity of this argument being affected. It follows that any change in the amount of P^{32} in a given nucleotide during bacterial multiplication is accurately reflected in a change in specific activity of that nucleotide using the carrier technique.

For each of the three experiments the average results for the four ribonucleotides and the four desoxyribonucleotides shown in Table I were plotted against time in Fig. 1. The data shown in Table I are actually specific

TABLE I

METABOLIC STABILITY OF NUCLEOTIDE PHOSPHORUS IN *E. coli*

Expt.	Time, hr.	Specific activities of RNA nucleotides,* counts/min./μgm. P					Specific activities of DNA nucleotides,* counts/min./μgm. P				
		Adenylic	Guanylic	Cytidylic	Uridylic	Average	Adenylic	Guanylic	Cytidylic	Thymidylic	Average
1	0	1400	1700	—	1700	1600	1400	1600	1600	1700	1600
	2.25	1300	1300	1600	1400	1400	1400	1600	2000	1400	1600
	4.50	1100	1100	1400	1100	1200	1400	1400	1700	1300	1500
2	0	1600	1600	1600	1700	1600	1500	1600	1800	1300	1600
	2.25	1600	1700	1600	1600	1600	1500	1600	1800	1600	1600
	4.50	1400	1300	1500	1600	1500	1500	2000	2300	1900	1900
3	0	1400	1500	1800	1600	1600					
	2.25	1600	1500	1500	1700	1600					
	4.50	1900	1400	1700	1600	1700					

**Specific activities of the separated nucleotides after addition of carrier bacteria to samples of experimental culture. The experimental data have been normalized in the following way: Averages were computed for the specific activities of the four RNA and the four DNA nucleotides at each time for each experiment. The average specific activity at zero time in Experiment I for the RNA nucleotides was found to be 1600 counts/min./μgm. P. The remaining zero time averages for both RNA and DNA nucleotides in the three experiments were then assumed to be 1600 counts/min./μgm. P. All the corresponding experimental results were normalized to this figure and entered into the table. As an example, the average of the actual experimental results for DNA nucleotides at zero time in Experiment 2 was 850 counts/min./μgm. P. Each of the results in Experiment 2 for DNA nucleotides was therefore multiplied by the factor 1600/850. Normalization was carried out in this way to facilitate comparison of one experiment to another and demonstrate more readily any trends in the results.*

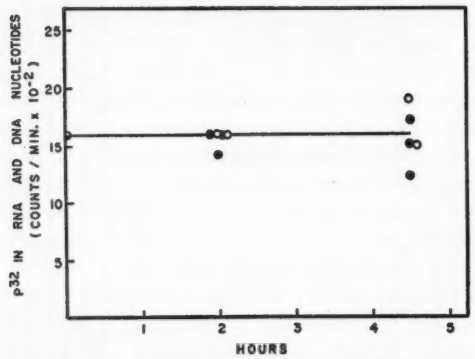

FIG. 1. Retention of nucleotide P^{32} in the RNA and DNA of labelled *E. coli* during multiplication in isotope free medium. The average results for RNA and DNA nucleotides shown in Table I have been plotted against time. As explained in the text the ordinates represent the amounts of P^{32} in each bacterial nucleic acid per unit volume of experimental culture. Closed circles represent RNA, open circles DNA.

activities but, for the reasons given above, represent, and are plotted in Fig. 1 as, amounts of P^{32} in each nucleic acid per unit volume of experimental culture. It is evident that there is no significant loss of radioactivity from either RNA or DNA nucleotides during several generations of bacterial multiplication. This is additional evidence, and in agreement with that of Hershey (2), that, once synthesized, there is no net loss of P^{32} from either nucleic acid. The above experiments demonstrate also that there is little or no unequal exchange of P^{32} between the nucleotides of either nucleic acid during bacterial multiplication.

Recently Stevens *et al.* (14) have postulated that, for several types of animal cell, the DNA of the mother cell is broken down during mitosis and the DNA of the two daughter cells is synthesized *de novo*. Their hypothesis predicts that P^{32} incorporated into DNA will be lost from it during subsequent replication in isotope free medium. Our results, and those of Hershey (2), show that replication of DNA in *E. coli*, during the logarithmic phase of multiplication, does not involve such a mechanism.

Incorporation of Inorganic Phosphorus into Bacterial Nucleic Acids from Medium of Constant Specific Activity

The foregoing experiments suggest that half the nucleic acids of the daughter cells are derived from the mother organism and half are newly synthesized. To investigate this point in more detail, the kinetics of phosphorus assimilation into bacterial RNA and DNA, from a medium of constant specific activity, were measured. In this experiment inorganic P^{32} was added to a 10 ml. culture of *E. coli* in the logarithmic phase and containing 8×10^7 cells per ml. At intervals during subsequent multiplication of the bacteria, 1 ml. aliquots were removed and treated through the alkaline digestion step for nucleic acid separation. At this point equal volumes of potassium hydroxide treated carrier

TABLE II

INCORPORATION OF P^{32} INTO RNA AND DNA OF *E. coli* FROM MEDIUM OF CONSTANT SPECIFIC ACTIVITY

Time after addition of P^{32}, min.	Optical density	Measured specific activities of RNA nucleotides,* counts/min./μgm. P					Average relative specific activities of RNA nucleotides†	Measured specific activities of DNA nucleotides,* counts/min./μgm. P					Average relative specific activities of DNA nucleotides†
		Adenylic	Guanylic	Cytidylic	Uridylic	Average		Adenylic	Guanylic	Cytidylic	Thymidylic	Average	
5	6.2	17	14	22	15	17	2.7	5.0	3.7	5.0	—	4.6	0.7
10	6.7	39	37	42	50	42	6.2	17	21	15	17	18	2.6
25	8.4	200	180	210	210	200	23	110	160	84	94	110	13
50	12.3	590	580	630	650	610	50	360	380	310	300	340	27
97	25.5	2000	1900	2100	1900	2000	77	1400	1500	1400	1700	1500	59
200	105.5	8500	10000	11000	9100	9700	92	8500	7900	8900	—	8400	80

**Specific activities of the separated nucleotides after addition of carrier bacteria to samples of experimental culture.*

†Relative specific activity $= \frac{\textit{measured specific activity of nucleotide}}{\textit{optical density of culture}}$.

cells (see previous section) were added to each sample. The amount of carrier cell material added to each sample was at least one hundred fold in excess of that from the experimental bacteria. Mononucleotides of RNA and DNA were then separated and their specific activities determined. The results are shown in Table II.

As explained in the previous section such results bear no direct relationship to the actual specific activities of the nucleic acid nucleotides in the experimental culture, but they do represent the amount of P^{32} in these nucleotides. To obtain an estimate of the actual specific activities, we assume that the amount of bacterial nucleic acid phosphorus in a culture during the logarithmic growth phase increases at the same rate as the optical density and that the carrier bacteria have the same chemical composition as the bacteria in the experimental culture. Both assumptions are probably valid. If the measured specific activity for each nucleotide in Table II is divided by the optical density of the culture at the time the sample was taken, the figures thus derived are proportional to the specific activities of the RNA and DNA nucleotides at the various times in the experimental culture, and are therefore called *relative specific activities*. The average *relative specific activities* for the four RNA and four DNA nucleotides are shown in Table II and are plotted against time in Fig. 2 for the first two generations of bacteria.

It was desired to construct a hypothetical curve, for the increase in specific activities of the nucleic acids, based on the assumptions that there is no renewal of nucleic acid during bacterial multiplication and that there was no appreciable time required for inorganic phosphate to pass from the medium into the nucleic acids. Since the bacteria were grown in a medium of constant specific activity, the specific activities of the nucleic acids should then be 50% of the specific activities of inorganic phosphorus in the medium after one generation, 75% after two generations, etc. Because the *relative specific*

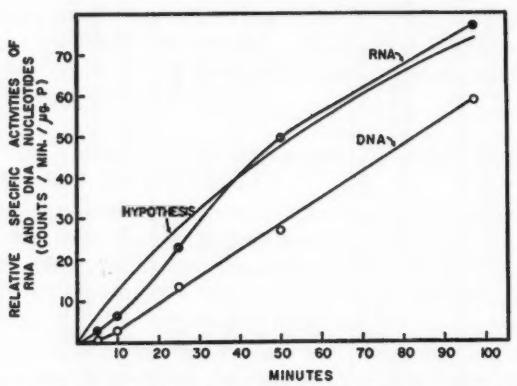

FIG. 2. Assimilation of inorganic P^{32} into the nucleotides of RNA and DNA during multiplication of *E. coli* in a medium of constant specific activity. The hypothetical curve is calculated according to the consideration given in the text.

activities were determined after the addition of carrier bacteria they could not be compared directly to the specific activity of the medium. Nevertheless, it was possible to derive an operational value for the specific activity of the medium which could be used to fix the position of the hypothetical curve and with which the *relative specific activities* of Fig. 2 could be compared. From separate experiments, in which no carrier bacteria were used, it was known that the phosphate of RNA achieved a specific activity approximately 90% of that of the medium in four bacterial generations. For the present experiment the *relative specific activities* for the four ribonucleotides after four generations could be derived from the last row of Table II. The average of these four results was assumed to represent 90% of the specific activity of the medium, and was used to calculate the hypothetical curve plotted in Fig. 2.

Fig. 2 shows that both experimental curves lag behind that predicted by the hypothesis. The lag suggests that considerable time might be required for inorganic phosphate from the medium to pass through the nucleic acid precursor pools, since it has been shown in the first experiment that there is no renewal of either nucleic acid. Moreover, it would be predicted from these curves that the DNAP precursor pool is larger than the RNAP precursor pool, and this prediction was verified by the experiment described in the following section.

Kinetics of Incorporation of Inorganic Phosphorus into Nucleic Acids

In this experiment a technique was used similar in principle to that devised by Stent and Maaloe (13). Bacteria in the logarithmic phase were grown for two minutes in a medium containing P^{32} of very high specific activity. Further significant uptake of P^{32} from the medium was then prevented, and the time for the phosphate taken up during the two minute interval to be incorporated into bacterial nucleic acids was measured.

A 10 ml. culture of *E. coli* was grown in medium containing 7 μgm. of phosphorus per ml. When the bacteria reached a density of 6×10^8 cells per ml. that is, when about 5 μgm. of the phosphorus had been utilized (6), 1 mc. of carrier-free inorganic P^{32} was added. After agitation for two minutes at 37° C. the culture was diluted 10-fold into medium of the original composition but containing 3000 μgm. of inorganic phosphorus per ml. By this means, uptake of P^{32} from the medium during subsequent bacterial multiplication was reduced to a negligible amount. Agitation of the culture at 37° C. was continued and aliquots were withdrawn at intervals. The bacteria remained in the logarithmic phase throughout and were not at any time starved for phosphorus. A large excess of carrier bacteria was added in equal amount to each sample at the alkaline digestion stage, as before. RNA and DNA nucleotides were then separated from each mixture and their specific activities measured. The results are shown in Table III.

For the reasons discussed under the first experiment the measured specific activities represent amounts of P^{32} contained in the nucleic acid nucleotides before addition of carrier. Although the RNA nucleotide results are comparable among themselves, as are the DNA nucleotide results, RNA and DNA

have undergone unequal dilution by the addition of carrier and the two sets of results cannot yet be compared to each other. Since the ratio of RNAP to DNAP for *E. coli* propagated under the present conditions is 4 (calculated from analyses of RNA and DNA bases in this laboratory (unpublished)), RNA in the experimental samples will have undergone greater dilution than DNA by addition of carrier. The experimentally determined specific activities for RNA nucleotides, shown in Table III, must therefore be increased by a factor of four to represent properly the radioactivities of RNA nucleotides relative to DNA nucleotides in the original culture. For each row of Table III the average of the four ribonucleotide results was multiplied by four, and these figures, with the averages of the four desoxyribonucleotide results, are plotted against time in Fig. 3. The curves of Fig. 3 now represent essentially the amount of nucleotide P^{32} in each nucleic acid per ml. of experimental culture, and are designated as such.

Fig. 3 shows that the P^{32} contents of both nucleic acids increase from zero time and tend to a constant value after about 50 to 60 min. It therefore requires at least one generation time to deplete the P^{32} from both nucleic acid precursor pools. By precursor pool is meant any intracellular phosphorus destined to become RNA or DNA that has not already done so. As mentioned earlier we have found that RNAP/DNAP = 4 for this strain of *E. coli*. Inspection of Fig. 3 shows that when incorporation of precursor P^{32} into nucleic acids was complete $RNAP^{32}/DNAP^{32} = 6$. Such a disproportionate amount of P^{32} in RNA relative to DNA might be observed if the ratio of RNA to DNA phosphorus in the carrier bacteria were different to that in the experimental culture. This possibility was eliminated by conducting an experiment similar to the one above but without the use of carrier for the

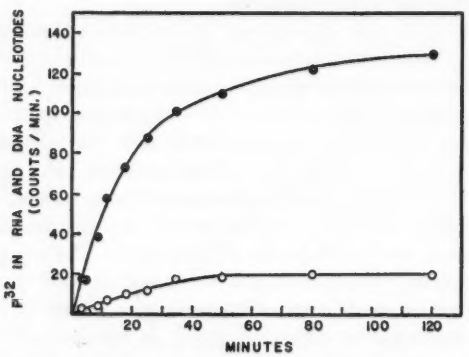

FIG. 3. Assimilation of inorganic P^{32} into the nucleotides of RNA and DNA during multiplication of *E. coli*. At time zero on the graph, bacteria, while in the logarithmic phase of growth, were exposed for two minutes to P^{32}. The curves show the time required for this two minute "pulse" of P^{32} to be incorporated into RNA and DNA. For the DNA uptake curve (open circles) the average results for DNA nucleotides, shown in Table III, were plotted. The averages for RNA nucleotides, shown in Table III, were each increased by a factor of 4, to allow for unequal dilution of RNA and DNA by carrier, as explained in the text, and then plotted as the RNA uptake curve (closed circles).

TABLE III

INCORPORATION OF P^{32} INTO RNA AND DNA OF *E. coli* AFTER ADDITION OF ISOTOPE FOR TWO MINUTES

Time after addition of P^{32}, min.	Measured specific activities of RNA nucleotides,* counts/min./μgm. P					Measured specific activities of DNA nucleotides,* counts/min./μgm. P				
	Adenylic	Guanylic	Cytidylic	Uridylic	Average	Adenylic	Guanylic	Cytidylic	Thymidylic	Average
3	3.0	6.5	3.3	4.2	4.3	5.8	1.6	0.3	2.4	2.5
5	3.4	3.1	4.3	7.1	4.5	1.0	2.0	0	0	0.8
8	11	7.4	7.8	12	9.6	0	5.7	1.4	5.2	3.1
12	18	13	13	14	15	10	7.5	4.6	5.6	6.9
18	16	20	15	20	18	11	11	8.7	8.7	9.9
25	20	20	23	23	22	14	13	11	10	12
35	27	19	27	28	25	17	17	—	19	18
50	29	25	27	30	28	21	19	18	17	19
80	28	30	33	31	31	23	20	20	19	21
120	30	35	33	32	33	21	20	21	21	21

**Specific activities of the separated nucleotides after addition of carrier bacteria to samples of experimental culture.*

nucleotide analyses. The ratio of P^{32} in RNA and DNA nucleotides was measured only at one point, 120 min., and found to be the same as shown in Fig. 3. RNA phosphorus has therefore finally attained a specific activity 50% greater than that of DNA phosphorus.

It is difficult to explain why the two minute "pulse" of P^{32}, introduced into bacteria already in a state of equilibrium as regards phosphate assimilation, should distribute unequally between RNA and DNA. An indication that phosphate is assimilated at different rates by RNA and DNA had, of course, been apparent already from Fig. 2. The unequal distribution of the P^{32} "pulse" might be explained on the assumption that the P^{32} is "trapped" by an early irreversible reaction in the RNA precursor pool while all the early reactions in the DNA precursor pool are reversible.

Since the curves of Fig. 3 show no tendency to approach each other after the P^{32} precursor pools have emptied, we conclude that if an equal exchange of phosphorus occurs between RNA and DNA it must be very small during one generation. Hershey's results (2) have already indicated that an unequal interchange of phosphorus does not occur. It is therefore apparent that the phosphorus of each nucleic acid and, in fact, of each nucleotide, is conserved during bacterial multiplication.

Another possibility has not yet been eliminated, namely, that one or both nucleic acids break down during multiplication, liberate phosphorus into their respective precursor pools, and then reutilize all this phosphorus without exchange having occurred between the pools. It is difficult to visualize an experiment that would settle this point.

From the curves of Fig. 3 the half-life of precursor phosphorus is about 14 minutes for RNA and 20 minutes for DNA. The amount of RNA phosphorus at time t is

$$R_t = R_0 e^{\alpha t} ,$$

where R_0 is the amount of RNA phosphorus at time zero, and α is the growth constant. For a bacterial generation time of 54 min. the amount of RNA phosphorus formed in 14 min. is then

$$R_{(14)} = 0.20\, R_0 .$$

This RNA phosphorus is formed from precursor phosphorus and half the precursor phosphorus disappears in 14 min. so

$$R_{(14)} = P_0/2 = 0.20\, R_0 \text{ or } P_0 = 0.40\, R_0 ,$$

where P_0 is equal to the amount of phosphorus in the precursor pool at time zero. The precursor pool for RNA phosphorus is, therefore, a little less than half the amount of RNA phosphorus already present. For DNA the phosphorus precursor pool size is equal to 0.59 of the DNA phosphorus present.

This experiment also illustrates two of the uncertainties involved in the calculation of turnover rates, a problem which has been discussed fully by Reiner (10). It is common practice in turnover studies on animal tissues to compare the turnover rates of the two nucleic acids from the relative rates of P^{32} uptake. The results shown in Fig. 3 demonstrate that *there may be a marked difference in the rates of assimilation of P^{32} into RNA and DNA even with a system in which significant nucleic acid turnover does not occur.* Further, it is clear from the considerations of Reiner (10) that if an isotopically labelled compound passes through a precursor pool of finite size before incorporation into a specific macromolecule, no measure of the kinetics of incorporation of the isotope into the macromolecule will allow calculation of its turnover rate. It is evident from our results that for phosphorus precursors of nucleic acids these pools can be relatively very large.

Acknowledgment

We are indebted to Mr. Peter Ove for technical assistance.

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EFFECTS OF SODIUM AND POTASSIUM CHLORIDE ON CERTAIN ENZYMES OF MICROCOCCUS HALODENITRIFICANS AND PSEUDOMONAS SALINARIA[1]

By R. M. Baxter and N. E. Gibbons

Abstract

The isocitric, succinic, malic, and lactic dehydrogenases and cytochrome oxidase from *Pseudomonas salinaria*, an extreme halophile, are most active in high concentrations of sodium or potassium chloride. The lactic dehydrogenase and cytochrome oxidase of *Micrococcus halodenitrificans* are also more active at high concentrations of salt, but the isocitric, succinic, malic, α-ketoglutaric, and glutamic dehydrogenases of this moderate halophile are most active at much lower concentrations. It is concluded that the extreme halophiles have a high intracellular salt content. It is suggested that *M. halodenitrificans* maintains an intracellular salt concentration of about 0.5 *M* under optimal conditions for growth and that cytochrome oxidase is important in maintaining this level against a concentration gradient.

Introduction

Most halophilic bacteria fall into one of two groups on the basis of the sodium chloride concentration range in which they will grow. Moderate halophiles grow in concentrations of sodium chloride ranging from 1 or 2% to about 20%. Extreme halophiles do not grow at concentrations below about 15%, and grow even in saturated brines (about 31%, w/v).

In a previous paper (1) we reported that the glycerol dehydrogenase of *Pseudomonas salinaria*, an extreme halophile, required high concentrations of sodium chloride or potassium chloride for maximum activity, while the glycerol dehydrogenases of *Vibrio costicolus*, a moderate halophile, and of *Escherichia coli* were most active at rather low salt concentrations. About the same time Robinson and Katznelson (9) described a transaminase from *P. salinaria* which was most active at high salt concentrations.

Ingram (5) suggested that the proteins of halophiles may be less readily salted out than those of other bacteria. Robinson, Gibbons, and Thatcher (8), on the other hand, presented evidence that the moderate halophile *Micrococcus halodenitrificans* maintains a low intracellular salt concentration by an energy dependent mechanism. Our observations on glycerol dehydrogenases indicated that extreme halophiles may possess a high intracellular salt concentration, to which their enzymes are adapted, as suggested by Ingram, while moderate halophiles may maintain a low intracellular salt concentration, as suggested by Robinson, Gibbons, and Thatcher. A fundamental physiological difference thus appeared to exist between the two groups of organisms.

Shortly after our paper (1) appeared, the work of Egami and his co-workers (4, 15) was brought to our attention. These authors described an alkaline phosphomonoesterase from a moderate halophile, designated as *Pseudomonas* No. 101, which resembled the glycerol dehydrogenase of *P. salinaria* in being

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most active at high salt concentrations. Later reports from Egami's laboratory described a salt-requiring glucose dehydrogenase from the same organism (14) and a salt-requiring cytochrome oxidase from a halotolerant micrococcus (13). Several other enzymes from the organisms studied by the Japanese workers do not require salt (3, 4, 14).

These facts suggested that the physiological difference between moderate and extreme halophiles may be less sharp than we had proposed. This paper describes the effects of salts on several enzymes from a moderate halophile, *Micrococcus halodenitrificans*, and several from *P. salinaria* in an attempt to assess the importance of salt-requiring enzymes in the adaptation of these organisms to life in brines.

Materials and Methods

Pseudomonas salinaria was grown as described previously (1). *Micrococcus halodenitrificans* was grown at room temperature on 0.5% proteose peptone and tryptone medium containing 8% sodium chloride. Cells were harvested in a Sharples supercentrifuge, washed with and finally suspended in sodium chloride solution of the same concentration as the growth medium, and broken by sonic vibration using a Raytheon 10 kilocycle sonic oscillator. The extracts were centrifuged in an M.S.E. high speed centrifuge at 10,000–11,000 r.p.m. and the supernatant stored at 4°–5° C. No attempt was made to purify any of the enzymes studied since partial purification of the glycerol dehydrogenase of *P. salinaria* (1) or of the halophilic phosphatase studied by Egami and his group (3) did not change the response of these enzymes to salt.

The activities of dehydrogenases not linked to DPN[2] or TPN[2] were measured manometrically in the Warburg apparatus by the ferricyanide technique of Quastel and Wheatley (6). The output of carbon dioxide in 30 min., corrected for the output in the absence of substrate, was used as a measure of enzyme activity. Substrate concentrations ranged from 0.003 to 0.01 *M*, the concentration chosen for each substrate being that found to give maximal activity. In the measurement of α-ketoglutaric dehydrogenase activity each vessel contained 0.5 mgm. TPP. The activity of the malic dehydrogenase of *P. salinaria* was also measured by the ferricyanide technique, in the presence of 1.0 mgm. DPN. All the enzymes of *M. halodenitrificans* were studied at 36° C., except the α-ketoglutaric dehydrogenase, which was studied at 29.5°. The enzymes of *P. salinaria* were also studied at 29.5° because the activity of the enzymes of this organism declined rather rapidly with time at the higher temperature. Experiments on the glycerol dehydrogenase of *P. salinaria* (unpublished) indicated that the salt concentration for maximal activity increased with increasing temperature. The effect of the difference in temperature will be to decrease rather than increase the difference between the enzymes of the two organisms.

[2]*The following abbreviations are used: DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; TPP, thiamine pyrophosphate. DPN and TPN were obtained from the Sigma Chemical Company and TPP from Nutritional Biochemicals Corporation.*

The activities of malic dehydrogenase of *M. halodenitrificans*, and the isocitric dehydrogenases of both organisms were measured by following the increase in reduced DPN or TPN, as indicated by the increase in optical density at 340 mμ in the Beckman spectrophotometer. The increase in optical density in five minutes was taken as a measure of enzyme activity. Phosphate buffer (*M*/30, pH 7.3) was used with the isocitric dehydrogenase of *M. halodenitrificans*, and hydrazine buffer (0.3 *M*, pH 7.3) with the other enzymes.

Cytochrome oxidase activity was determined by measuring the oxygen uptake in the Warburg apparatus in the presence of *p*-phenylenediamine, 10 mgm./ml. The oxygen uptake in 30 min., corrected for the autoxidation of *p*-phenylenediamine, was taken as a measure of enzyme activity.

Results

Tricarboxylic Acid Cycle Dehydrogenases

The four dehydrogenases of the tricarboxylic acid cycle were present in extracts of *Micrococcus halodenitrificans*, but in *Pseudomonas salinaria* only three, succinic, malic, and isocitric dehydrogenases, could be demonstrated. Apart from their responses to salt, analogous enzymes from the two organisms were similar, and showed no unusual properties. Both malic dehydrogenases specifically required DPN, and both isocitric dehydrogenases TPN. The isocitric dehydrogenase of *M. halodenitrificans* required magnesium ion, but the corresponding enzyme from *P. salinaria* was fully active without the addition of magnesium. Possibly this enzyme contains sufficient magnesium for maximal activity in a firmly bound state. Neither succinic dehydrogenase could be shown to require any cofactor. The α-ketoglutaric dehydrogenase of *M. halodenitrificans* required TPP.

The effects of salts on corresponding enzymes from the two organisms are strikingly different (Fig. 1). Optimal salt concentrations for the enzyme of *M. halodenitrificans* ranged from less than 0.01 *M* (malic dehydrogenase) to about 0.6 *M* (α-ketoglutaric dehydrogenase), and there was little difference between the effects of sodium chloride and potassium chloride. The enzymes from *P. salinaria* on the other hand were most active in the presence of potassium chloride at concentrations of about 3 *M*, or higher. Sodium chloride was much less effective than potassium chloride in activating all the enzymes except malic dehydrogenase.

Other Dehydrogenases

M. halodenitrificans was found to contain a glutamic dehydrogenase which did not appear to require a coenzyme. It is most active at a salt concentration of about 0.3 *M* (Fig. 2). A corresponding enzyme could not be demonstrated in *P. salinaria*.

Both organisms were found to contain lactic dehydrogenases which do not require coenzymes. That from *P. salinaria* was similar to the other dehydrogenases of this organism (Fig. 2). The lactic dehydrogenase from *M. halodeni-*

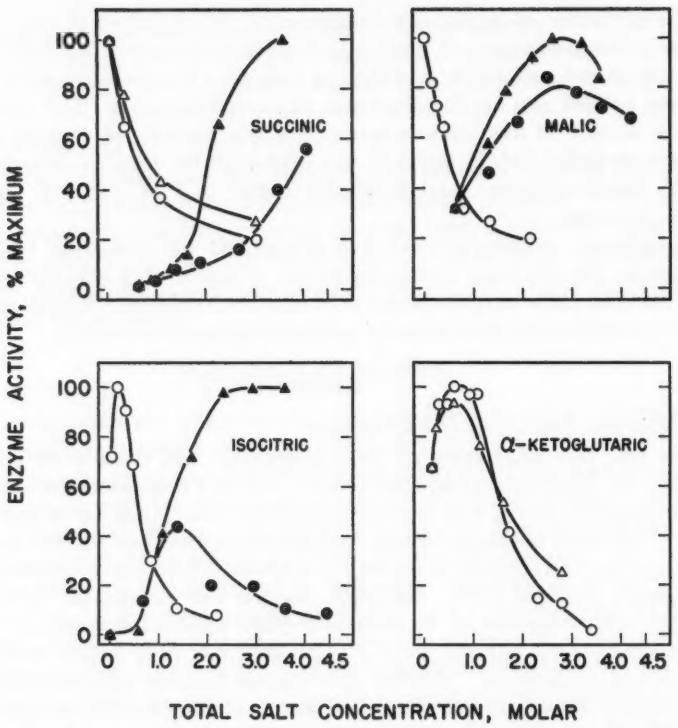

FIG. 1. Effects of sodium chloride and potassium chloride on the tricarboxylic acid cycle dehydrogenases of *Micrococcus halodenitrificans* and *Pseudomonas salinaria*. ○: *M. halodenitrificans*, NaCl; △: *M. halodenitrificans*, KCl; ●: *P. salinaria*, NaCl; ▲: *P. salinaria*, KCl.

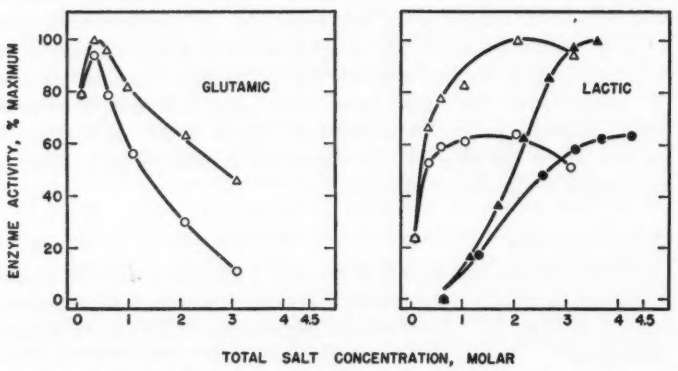

FIG. 2. Effects of sodium chloride and potassium chloride on glutamic and lactic dehydrogenase of *Micrococcus halodenitrificans*, and lactic dehydrogenase of *Pseudomonas salinaria*. ○: *M. halodenitrificans*, NaCl; △: *M. halodenitrificans*, KCl; ●: *P. salinaria*, NaCl; ▲: *P. salinaria*, KCl.

trificans (Fig. 2) was markedly different from the other dehydrogenases of the micrococcus, showing optimal activity at a potassium chloride concentration of about 2 *M* and little decline in activity even at 3 *M*. Sodium chloride was much less effective than potassium chloride in activating the enzymes of both organisms.

Cytochrome Oxidase

The cytochrome oxidases of both organisms were found to be halophilic, and differed from most other halophilic enzymes in being activated equally by either sodium or potassium chloride (Fig. 3). The responses of the two enzymes to salt were nearly the same, except that the enzyme from *M. halodenitrificans* showed relatively greater activity at low salt concentrations.

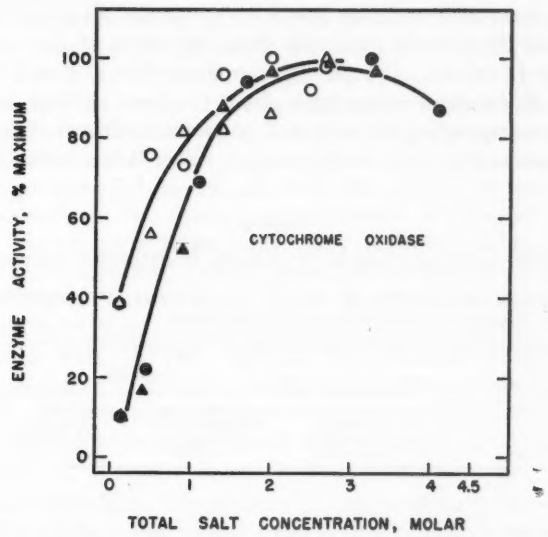

FIG. 3. Effects of sodium chloride and potassium chloride on cytochrome oxidase of *Micrococcus halodenitrificans* and *Pseudomonas salinaria*. ○: *M. halodenitrificans*, NaCl; △: *M. halodenitrificans*, KCl; ●: *P. salinaria*, NaCl; ▲: *P. salinaria*, KCl.

Discussion

Bacterial halophilism involves two phenomena, which are not necessarily related: tolerance of salt below a certain maximum and a requirement for salt above a certain minimum. There is evidence (10) that at low salt concentrations the permeability of the cell wall or cytoplasmic membrane of moderate halophiles is greatly increased allowing leakage of cytoplasmic constituents. Certain of the extreme halophiles, particularly rod-shaped forms, appear to dissolve completely at low salt concentrations. These observations provide at least a partial explanation of the salt requirement of

halophiles. In extreme halophiles, salt is apparently also required for the activity of many, if not all, of the enzymes of the organism. Seven enzymes of *P. salinaria* have been described and all are halophilic (Table I).

The observation that the enzymes of *P. salinaria* are most active at high salt concentrations provides at the same time a partial explanation of the salt tolerance of the organism. Other macromolecular constituents of the organism may be similarly adapted to high salt concentrations. In particular the chromatin material may well differ from that of non-halophiles, which has been shown to be very sensitive to changes in the ionic environment (12). There seems to be no reason to modify our earlier view (1) that this organism possesses a high intracellular electrolyte concentration to which its vital processes are adapted, and that this is probably a general characteristic of extreme halophiles.

The salt tolerance of moderate halophiles cannot be adequately explained on this basis. Of 15 enzymes studied in three organisms of this type, only four are halophilic (Table I). It is perhaps significant that although the halophilic enzymes of *M. halodenitrificans* have about the same optimal salt concentrations as the corresponding enzymes of *P. salinaria*, their activity at suboptimal concentrations is relatively much greater. This is particularly true of the

TABLE I

THE SALT REQUIREMENTS OF ENZYMES OF HALOPHILIC BACTERIA

H: Optimal salt concentration $\geq$ 1.5 *M*. N: Optimal salt concentration $\leq$ 0.5 *M*
0: Not found. Figures in parentheses refer to references.

Enzyme	Organism			
	Pseudomonas salinaria (Extreme halophile)	*Micrococcus halodenitrificans* (Moderate halophile)	*Vibrio costicolus* (Moderate halophile)	*Pseudomonas* No. 101 (4) (Moderate halophile)
Dehydrogenases				
Isocitric	H	N	—	—
α-Ketoglutaric	0	N	—	—
Succinic	H	N	—	—
Malic	H	N	—	—
Lactic	H	H	—	—
Glucose	0	—	—	H (14)
Formic	0	0	—	N (14)
Glycerol	H (1)	0	N (1)	—
Glutamic	0	N	—	—
Nitrite reductase – lactic dehydrogenase	—	N (8)	—	—
Nitrate reductase – succinoxidase	—	—	N (7)	—
Cytochrome oxidase	H	H	—	—
Transaminase	H (9)	—	—	—
Protease	—	—	—	N (4)
Catalase	—	—	—	N (4)
Alkaline phosphomonoesterase	—	—	—	N (4)

lactic dehydrogenase (Fig. 2). At a salt concentration of 0.5 *M*, none of the halophilic or non-halophilic enzymes of *M. halodenitrificans* described in this paper operates at less than 55% of its maximum activity.

It also seems significant that one of the halophilic enzymes is cytochrome oxidase. It has often been postulated that the cytochrome system plays a role in ionic transfer in many organisms (e.g. (2)). If it is involved in ion transfer in *M. halodenitrificans*, the increase in its activity with increasing salt concentration may perhaps be regarded as a feedback mechanism tending to maintain a relatively constant internal environment. This also seems to be a reasonable explanation for the halophilic behavior of the cytochrome oxidase from a halotolerant organism (13), which would not be expected to contain a high concentration of salt.

On the basis of the foregoing discussion, the following seems a reasonable description of the behavior of *M. halodenitrificans*. Under optimal growth conditions, i.e. with 1 to 1.5 *M* salt in the medium, the cells contain about 0.5 *M* salt. At this concentration the various enzymes act at rates of from 60 to 100% of their maximum. The concentration of the various enzymes is probably such that under these conditions there is a maximal over-all rate of utilization of substrates. When salt enters the cell, driven by the steep concentration gradient across the cell wall, the activity of the cytochrome oxidase increases and the optimal concentration is restored. When the external salt concentration is too high, the cytochrome pump is unable to maintain the optimal internal concentration and a steady state is set up at a higher intracellular salt concentration where the activity of the non-halophilic dehydrogenases is inhibited with an over-all reduction in the rate of substrate utilization and growth. At very high salt concentrations the activity of the halophilic dehydrogenases and even of the cytochrome oxidase is reduced, so that eventually the maximum salt concentration at which growth may occur is reached when the cytochrome pump can no longer cope with the salt gradient.

The moderate halophiles then seem to be intermediate in the evolution of the extreme halophiles. For certain relatively halotolerant organisms saline environments provided an ecological niche in which potential competitors were unable to grow (11). The selection of mutants possessing a more halophilic cytochrome system and consequently a more efficient salt-transporting system gave rise to still more halotolerant forms. Further mutations which altered the cell walls and possibly other cellular components gave rise to moderate halophiles. However, in more concentrated brines, the expenditure of much energy was required to maintain a low internal concentration and eventually those forms were selected that could tolerate higher internal salt concentrations and thus conserve energy. Further mutation gave rise to the extreme halophiles in which all the enzymes are adapted to a high electrolyte concentration and the ion transporting mechanism is required only to concentrate potassium within the cell and to maintain very small differences in internal and external osmotic pressure.

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UTILIZATION OF SODIUM CASEINATE BY LACTIC STREPTOCOCCI AND ENTEROCOCCI[1]

By I. J. McDonald

Abstract

The ability of lactic streptococci to utilize unhydrolyzed sodium caseinate as the nitrogen source in an otherwise chemically defined medium seems to be a strain rather than a species characteristic. This characteristic therefore appears to have little value as an aid in classification although it might have some importance in selection of starter cultures for cheese manufacture. None of the enterococci examined grew with unhydrolyzed sodium caseinate as the sole nitrogen source. However, *Streptococcus liquefaciens* and *Streptococcus zymogenes* appeared to utilize sodium caseinate when small amounts of casein hydrolyzate were added to the medium.

Introduction

The ability of lactic streptococci to increase the soluble nitrogen in milk cultures has been demonstrated (6, 11) and the caseolytic properties of cultures of *Streptococcus liquefaciens* are well known. Lactic streptococci produce intracellular enzymes capable of hydrolyzing casein (2, 3, 12) and other investigations showed that growth of some strains of *Streptococcus lactis* in milk serum was enhanced by the addition of sodium caseinate (5). The present studies were undertaken to determine if lactic streptococci and enterococci could utilize sodium caseinate as a source of amino acids in an otherwise complete, chemically defined medium.

Materials and Methods

Cultures of lactic streptococci* were maintained by weekly transfer in milk containing 0.5% glucose and 0.5% yeast extract (Difco). Classification of cultures (1, 7, 8) indicated that 829, 7962, and 7963 were strains of *S. lactis* and the remainder were strains of *S. cremoris*. Cultures of enterococci† were maintained by weekly transfer in tryptone-tryptose agar (Medium II (4)).

Utilization of unhydrolyzed sodium caseinate by the test organisms was determined in the basal medium described previously (Medium I (4)) modified by the addition of 0.1 mgm. of unheated glutamine per ml. and by the omission of cysteine hydrochloride. Two per cent solutions of sodium caseinate (Nutritional Biochemicals Corp.) and pancreatin hydrolyzed sodium caseinate were prepared and sterilized by passage through a Seitz filter as described previously (4).

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Contribution from the Division of Applied Biology, National Research Laboratories, Ottawa, Canada. Issued as N.R.C. No. 4070.

**Strains 7962, 7963, 9596, and 9625 were from A.T.C.C.; 829 from the Bacteriology Division, Science Service, Ottawa; and the various HP, HLC, H22 cultures were isolated from commercial dairy starter cultures.*

†Streptococcus faecalis 9790, Streptococcus liquefaciens 799, and Streptococcus zymogenes 6054 were from A.T.C.C. Streptococcus durans 626 was from the N.R.C. collection.

The medium without sodium caseinate or hydrolyzed caseinate was adjusted to pH 6.6 and autoclaved for 15 min. at 15 lb. pressure. Filtered solutions of sodium caseinate were added to the cooled medium.

Cells used as inocula were centrifuged from 18 to 24 hr. cultures in tryptone-tryptose broth (Medium II (4)), washed three times, and diluted to their original volume with .9% saline. An inoculum of 0.1 ml. was used per 10 ml. of medium. After incubation (lactic streptococci at 30° C., enterococci at 35° C.), growth was measured by titrating the lactic acid produced to the phenolphthalein end point. The results are reported as ml. of 0.1 *N* NaOH per 10 ml. of culture corrected for the titration of the uninoculated control.

Results

Of 22 strains of lactic streptococci tested four were consistently capable of growth with sodium caseinate as a source of amino acids, 11 failed to grow, and seven were variable (Table I). Of those that grew, one strain was classified as *S. lactis* and three as *S. cremoris*; of those that did not grow, two strains were classified as *S. lactis* and nine as *S. cremoris*. The seven variable cultures (i.e. 9596, 9625, and HP strains) were classified as *S. cremoris*. All strains tested grew when pancreatin hydrolyzed caseinate was the nitrogen source. Final growth of the *caseinate utilizing* lactic streptococci was about the same whether unhydrolyzed sodium caseinate or pancreatin hydrolyzed caseinate was the nitrogen source. The rate of growth, however, was faster with the hydrolyzed than with the unhydrolyzed sodium caseinate. Growth with hydrolyzed caseinate did not appear to be related to the ability of strains to utilize unhydrolyzed sodium caseinate. Some strains that grew well with hydrolyzate failed to grow with sodium caseinate, whereas other strains that grew relatively poorly with hydrolyzate did grow with sodium caseinate (Table I).

TABLE I

ACID PRODUCTION BY LACTIC STREPTOCOCCI WITH SODIUM CASEINATE AND PANCREATIN HYDROLYZED SODIUM CASEINATE

(ml. 0.1 *N* NaOH/10 ml. culture after 65 hr. at 30° C.)

Organism	Sodium caseinate*	Hydrolyzed caseinate*	Organism	Sodium caseinate*	Hydrolyzed caseinate*
S. lactis			*S. cremoris*		
829	7.6	7.6	HLC-2	0.4	4.0
7962	0.6	6.6	HLC-3	0.4	6.2
7963	0.6	6.0	HLC-4	0.5	2.8
			HLC-5	0.6	2.8
S. cremoris			HLC-6	0.4	3.0
H22-7	0.4	2.4	HP-1	Variable	1.7
H22-8	0.4	3.1	HP-2	"	1.8
H22-9	0.5	3.1	HP-3	"	2.0
H22-10	2.1	2.4	HP-5	"	1.8
H22-11	1.9	2.6	HP-6	"	2.2
H22-12	2.6	2.4	9596	"	3.6
HLC-1	0.4	4.8	9625	"	3.5

**Basal medium (see text) + sodium caseinate (2.0 mgm./ml.) or pancreatin hydrolyzed sodium caseinate (equivalent to 2.0 mgm. sodium caseinate/ml.).*

TABLE II

ACID PRODUCTION BY ENTEROCOCCI WITH SODIUM CASEINATE AND PANCREATIN HYDROLYZED SODIUM CASEINATE

(ml. 0.1 *N* NaOH/10 ml. culture after 65 hr. at 35° C.)

Additions to basal*	*S. liquefaciens* 799	*S. zymogenes* 6054	*S. faecalis* 9790	*S. durans* 626
Pancreatin hydrolyzed caseinate (0.27 mgm. N/ml.)	3.1	4.0	3.4	4.2
Pancreatin hydrolyzed caseinate (0.03 mgm. N/ml.)	0.9	1.3	1.3	1.0
Sodium caseinate (0.27 mgm. N/ml.)	0.1	0.2	0.1	0.0
Sodium caseinate (0.27 mgm. N/ml.) + pancreatin hydrolyzed caseinate (0.03 mgm. N/ml.)	1.5	3.0	1.5	1.2

**Basal medium* (*see text*).

In synthetic media containing limited nutrients, carbon dioxide is known to stimulate growth of some organisms (10). However, incubation of lactic streptococci under an atmosphere of 95% nitrogen and 5% carbon dioxide or in the presence of sodium bicarbonate (0.42 mgm./ml.) did not affect their growth with sodium caseinate.

Growth of enterococci was obtained when pancreatin hydrolyzed sodium caseinate was the nitrogen source but was limited when a low concentration of hydrolyzate was present (Table II). When unhydrolyzed sodium caseinate was the nitrogen source, growth did not occur. *S. liquefaciens* and *S. zymogenes* grew better in the presence of sodium caseinate plus the low concentration of hydrolyzate than in the presence of either of these nitrogen sources alone. The growth of *S. faecalis* and *S. durans*, however, was the same with both nitrogen sources together as it was with only a low concentration of hydrolyzate. Thus *S. liquefaciens* and *S. zymogenes* appeared to utilize sodium caseinate in the presence but not in the absence of soluble nitrogen while *S. faecalis* and *S. durans* apparently were unable to utilize caseinate even in the presence of soluble nitrogen.

Discussion

The ability of lactic streptococci to utilize unhydrolyzed sodium caseinate appeared to be a strain rather than a species characteristic. As such, it appears to be of little value as an aid in the classification of *S. lactis* and *S. cremoris*.

Growth in a medium with unhydrolyzed sodium caseinate as a source of amino acids suggests that these organisms produced an extracellular protease. Previous investigators (2, 3, 12) have demonstrated the existence of intracellular protease in lactic streptococci and have discussed the possible relationship of these enzymes to cheese ripening (3, 9, 12). However, it appears that lactic streptococci might also contribute extracellular enzyme to the over-all cheese proteinase activity. Thus, caseinate utilization might be of some importance in the selection of starter cultures for cheese manufacture.

None of the species of enterococci examined was able to utilize unhydrolyzed sodium caseinate as a sole source of amino acids. In the presence of a low concentration of hydrolyzed caseinate, *S. liquefaciens* and *S. zymogenes* appeared to utilize sodium caseinate. These organisms, therefore, apparently produced protease as a result of growth with soluble nitrogen. *S. faecalis* and *S. durans*, however, did not utilize sodium caseinate even in the presence of soluble nitrogen and, therefore, apparently did not produce any proteolytic enzymes under these conditions.

Acknowledgment

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OBSERVATIONS ON THE METABOLIC ACTIVITY OF THE SOIL MICROFLORA[1]

By H. Katznelson and I. L. Stevenson

Abstract

The metabolic activity of the soil microflora has been studied manometrically with intact soil at optimum moisture and in the presence of various substrates. It was found that casamino acids were oxidized rapidly after a preliminary lag period. Oxygen uptake with this substrate showed no marked variation when the moisture level of the soil was between 60 and 80% of its moisture holding capacity. Mixtures of various non-nitrogenous substrates such as hexoses, pentoses, disaccharides, and organic acids yielded variable results. Uptake of oxygen by the soil in the presence of casamino acids was completely inhibited by dinitrophenol and azide. A marked decrease in respiratory activity in the soil was observed also when the reaction was lowered to pH 3.5 and 4.5. Considerable variation in gas exchange was obtained with soils which varied widely in fertility, organic matter, and nitrogen content.

Introduction

The necessity for studying soil as an ecological unit for a better understanding of the normal activity of the soil population was frequently emphasized by Winogradsky (16). Although some work was carried out along these lines in the intervening years, major attention was focused on the behavior of specific soil isolates. The recent advent of antibiotics and the subsequent study of soil antagonists in their natural habitat has helped to redirect attention to microbial activity in soil. The introduction of the soil percolation technique by Lees and Quastel (6) with its application to various soil microbiological problems (1, 7, 14) exemplifies this trend in soil microbiology.

More recently the study of soil as a whole has involved the use of a variety of respirometers to measure CO_2 and O_2 exchange. In 1932 Smith and Brown (12) described a modified Barcroft differential manometer for the determination of CO_2 evolution in soil. These authors stated that the rates of O_2 uptake and CO_2 evolution of soils were in the ratio of 1 : 1 and suggested that the two might be used interchangeably, thus confirming Russell's (11) belief that the amount of O_2 absorbed is a reliable criterion of the total activity of soil microorganisms. In 1947 Webley (15) reported on the use of the Warburg manometric technique, and later, Lees (5) described a modified Haldane respirometer for determining O_2 uptake in soil. Various modifications of these manometric techniques have proved extremely useful in the study of the endogenous activity of the soil flora (2, 10), soil aeration (9), nitrification (8), decomposition of herbicides (3), and the effects of air-drying on soil (13).

The desirability of developing a reliable and practical method for the study of metabolic activity in soil has prompted a reinvestigation of the manometric technique. The methods adopted and the results obtained are presented in this paper.

[1]*Manuscript received June 20, 1956.*
Contribution No. 418 from the Bacteriology Division, Science Service, Canada Department of Agriculture, Ottawa.

Preliminary Experiments

Initial attempts to concentrate large numbers of soil organisms into a small volume and thus increase total gas exchange above that which might be expected from a few grams of soil were made in the following manner. Field soil was suspended in $M/50$ phosphate buffer at pH 6.0 (1 part soil : 1.6 parts buffer), and placed in a Waring blendor for one minute. The mixture was allowed to settle for four hours after which the supernatant fluid was siphoned off and centrifuged at 6000 r.p.m. for five minutes. The resulting sediment was resuspended in buffer so that 2 ml. was equivalent to 400 ml. supernatant. Two milliliters of this suspension was used per Warburg vessel and mixtures of various substrates placed in the side arms. Results of these experiments are given in Fig. 1, where the ready availability of casamino acids to the soil flora is evident. Oxygen uptake with this material was much greater and proceeded at a much faster rate than with the other substrate mixtures tested. Although the bacterial count had been increased from 68 million (initial soil count/gm.) to about 5 billion in the final 2 ml. of suspension, a corresponding increase in respiratory activity did not occur. Inasmuch as the final suspension used was extremely viscous and not readily aerated on shaking, it was felt that the lack of oxygen may have been a limiting factor in this system. Because of the time and effort involved in the preparation of these suspensions and the obvious lack of aeration, subsequent experiments were carried out with the soil itself.

Influence of Moisture

In the following studies, soils previously held in a moist condition were rapidly air-dried (30–60 min.) at room temperature with the aid of a fan, sieved, and the 0.5–1.0 mm. fraction collected. It has previously been shown with soils dried for a longer period (12–24 hr.) that anomalous respiratory patterns result during the first few hours following remoistening (13). Four grams of soil was added to each Warburg vessel. Water or a solution of substrate was then added directly to and distributed as uniformly as possible over the soil in the flask. Sufficient solution was used to bring the moisture content of the soil up to 20, 40, 60 and 80% of the moisture holding capacity (m.h.c.). Alkali was then added to the center wells, and the flasks attached to the manometers and placed in the water bath at 30° C. After a 30 min. equilibration period the manometers were closed and readings started. All tests were carried out in duplicate with casamino acids as the substrate.

The results illustrated in Fig. 2 indicate that oxygen uptake of the soil showed no marked variation when the moisture level was between 60 and 80% of its m.h.c. Below 60%, gas exchange decreased with decrease in moisture. A similar experiment carried out with another soil indicated comparatively little change in oxygen consumption between the 40 and 70% moisture levels. Although slight variation in the moisture levels necessary for optimum metabolic activity was noted for different soils, it would appear practical to standardize at the 60–70% level.

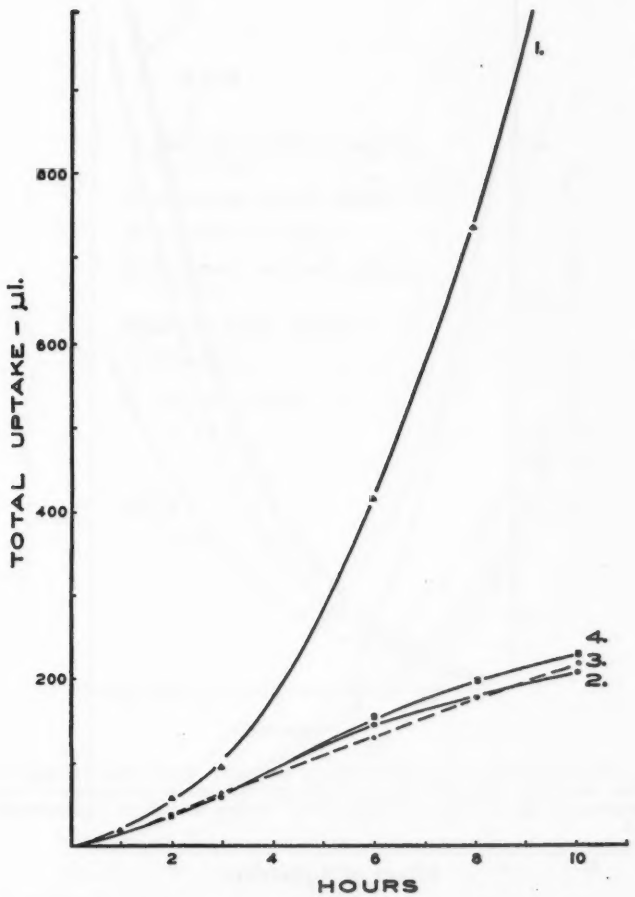

FIG. 1. Oxygen uptake by a concentrated soil suspension. Each Warburg vessel contained: 2.0 ml. soil concentrate, 0.5 ml. substrate, and 0.2 ml. 20% KOH. 1. Casamino acids, 10 mgm. 2. 0.1 ml. each of *M*/20 glucose, fructose, mannose, arabinose, xylose. 3. 0.1 ml. each of *M*/20 lactose, sucrose, maltose, glycerol, mannitol. 4. 0.1 ml. each of *M*/20 acetate, lactate, succinate, fumarate, pyruvate. Values for control (unamended) soil subtracted.

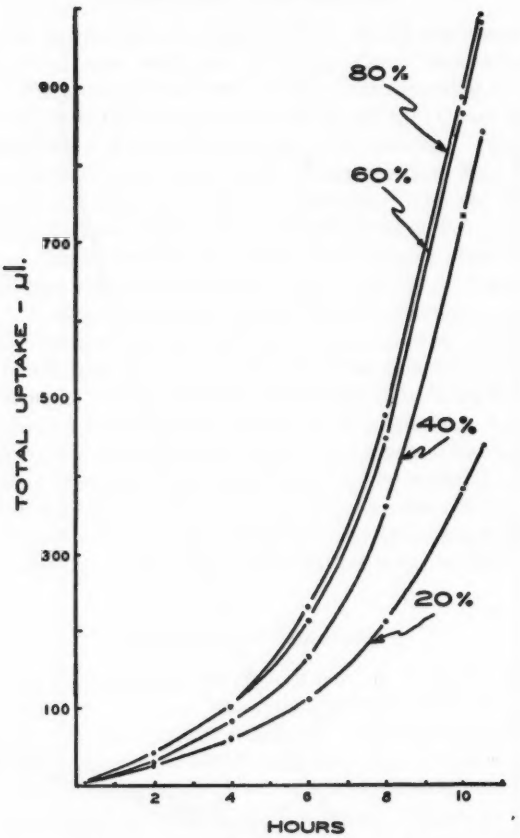

FIG. 2. Oxygen uptake by soil as affected by moisture. Each vessel contained 4 gm. soil, 0.5 ml. casamino acids (10 mgm.), water as indicated in percentage of moisture holding capacity of the soil, 0.2 ml. 20% KOH. Values for control (unamended) soils subtracted.

Effect of Substrate

In addition to casamino acids a number of other substrates were tested to determine the metabolic potential of the soil. Groups of hexoses, pentoses, disaccharides, and organic acids were used in the combinations indicated in Fig. 3. The mixture of all the carbon compounds gave an immediate and rapid response. Of the individual groups, that containing "acids" appeared to be superior to the others. Following a preliminary lag period casamino acids were again oxidized most rapidly. Glutamate oxidation followed a similar pattern except that a longer lag period was noted. When all the groups indicated were combined and one group at a time omitted, oxygen uptake was

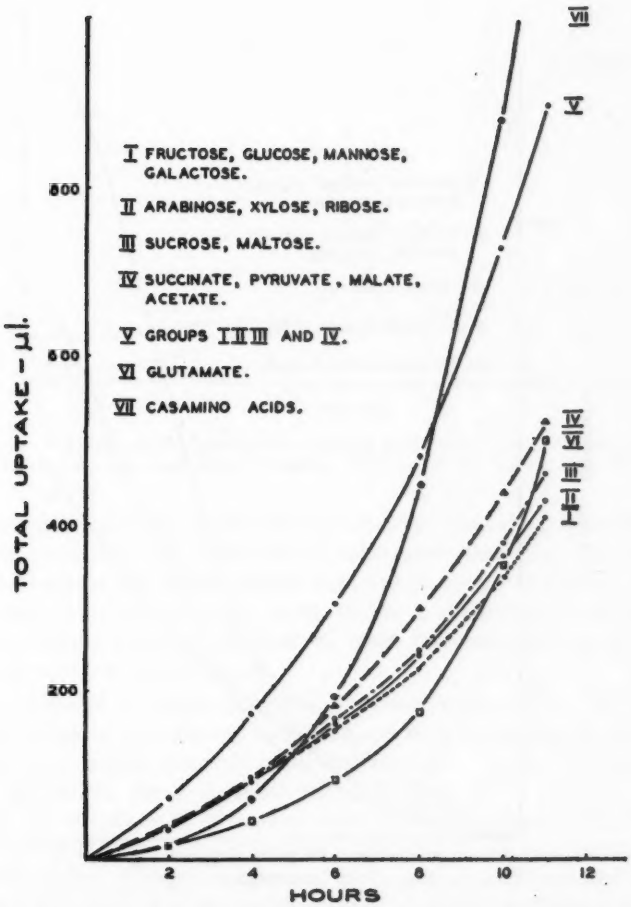

FIG. 3. Oxygen uptake with hexoses, pentoses, disaccharides, organic acids, glutamic acid, and casamino acids. Each vessel contained 4 gm. soil, 0.1 ml. *M*/10 non-nitrogenous substrates as indicated or 0.5 ml. *M*/20 glutamic or 0.5 ml. casamino acids, water to 1.5 ml., 0.2 ml. 20% KOH. Values for control (unamended) soil subtracted.

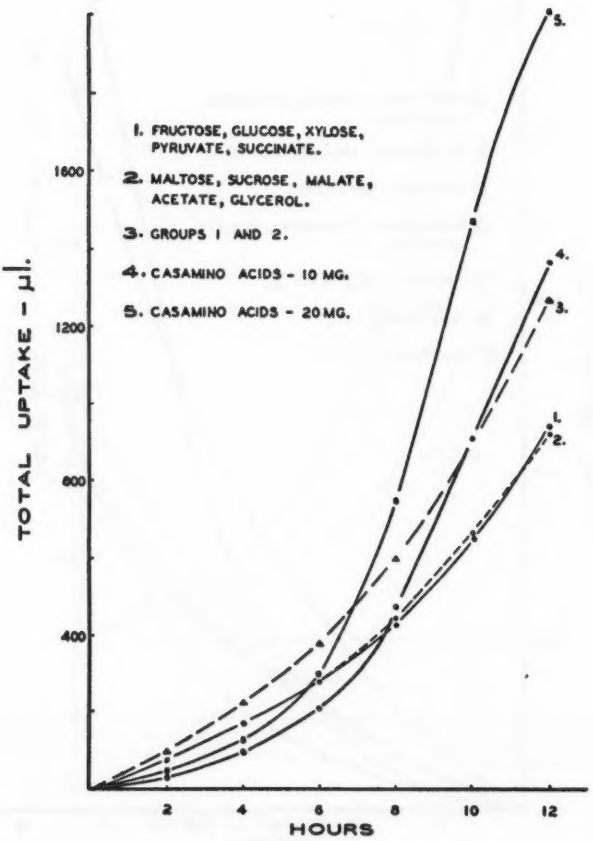

FIG. 4. Oxygen uptake with other mixtures of substrates and with two concentrations of casamino acids. Vessels contained 4 gm. soil, 0.1 ml. *M*/10 substrate as indicated or 0.5 ml. or 1.0 ml. casamino acids (10 or 20 mgm.), water to 1.5 ml., 0.2 ml. 20% KOH. Values for control (unamended) soil subtracted.

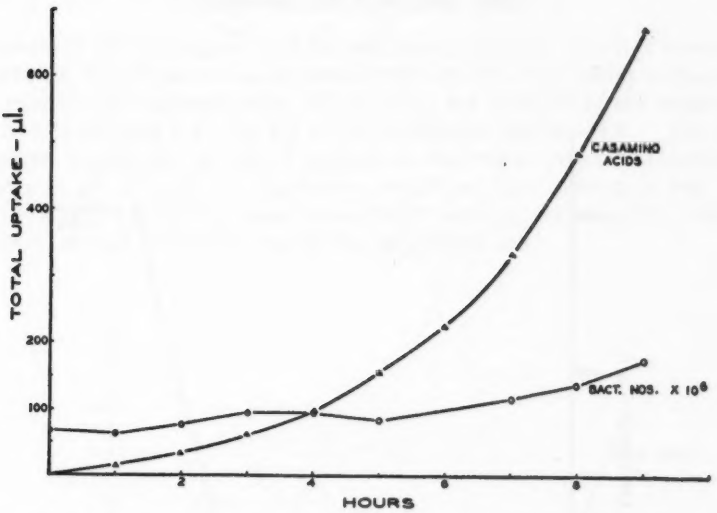

FIG. 5. Oxidation of casamino acids as related to bacterial count. Vessels contained 4 gm. soil, 0.5 ml. casamino acids (10 mgm.), water to 1.5 ml., 0.2 ml. 20% KOH.

lowered appreciably, the most pronounced effect occurring when the organic acids were left out. Combinations of substrates other than the above were tested and essentially similar results obtained as shown in Fig. 4. Doubling of the concentration of amino acids caused a distinct increase in oxygen uptake. A similar effect was observed when the concentration of the other carbon compounds was increased.

In an attempt to relate microbial numbers with oxygen uptake in the presence of casamino acids, soil was incubated with this substrate and samples were plated at hourly intervals in soil-extract agar. In the nine-hour period of the experiment the plate count increased from 60×10^6 to 172×10^6 whereas the hourly rate of oxygen consumption increased from 0 to 186. Direct counts of these samples showed a doubling of the total number of cells after nine hours. Oxygen uptake and plate counts are illustrated in Fig. 5. The results suggest that the rapid increase in oxygen utilization after a lag period of a number of hours may be due to an adaptive mechanism rather than to multiplication of soil bacteria. The introduction of inhibitors such as 2,4-dinitrophenol (DNP) and sodium azide in a final concentration of $M/2000$ completely inhibited oxygen uptake with casamino acids without appreciably affecting the activity of the unamended soil (Fig. 6). However, oxygen uptake was not inhibited when DNP was introduced four hours after the addition of casamino acids, further supporting the possibility that an adaptive mechanism was operative.

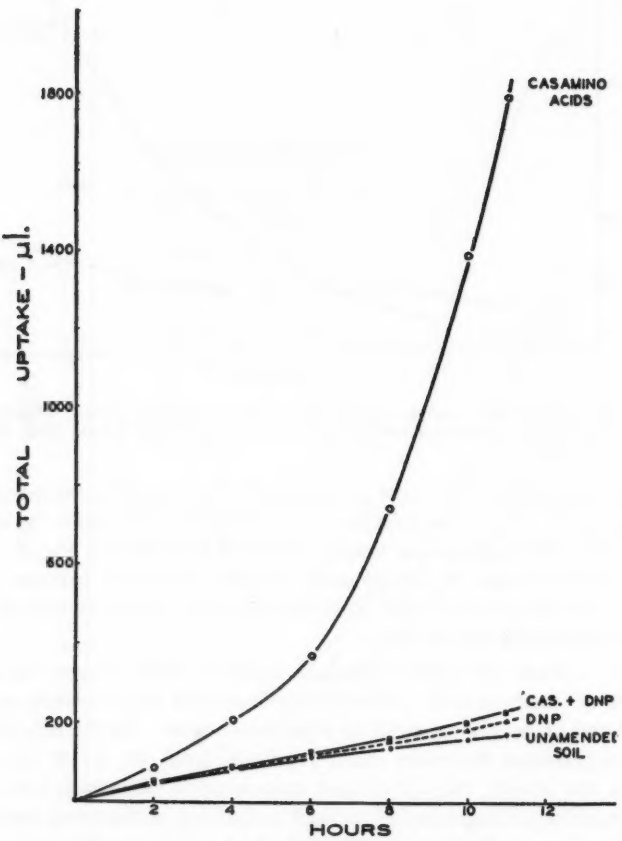

FIG. 6. Inhibition of casamino acid oxidation by 2,4-dinitrophenol. Vessels contained 4 gm. soil, 0.5 ml. casamino acids (20 mgm.), 0.3 ml. *M*/200 2,4-dinitrophenol, water to 1.5 ml., 0.2 ml. 20% KOH.

Influence of Reaction (pH)

In view of the heterogeneity of the soil micropopulation an effort was made to establish that proportion of the respiratory activity attributable to bacteria. Soil reaction was adjusted with HCl to bring the final pH of the moistened soil to 3.5, 4.5, and 5.5; the pH of the unadjusted soil was 6.8. The data presented graphically in Fig. 7 indicate a marked decrease in respiratory activity at pH 3.5 and 4.5, reactions at which bacterial activity is low. At pH 5.5, which is somewhat more favorable for bacteria, the metabolic activity is higher, though still lower than in the unacidified soil.

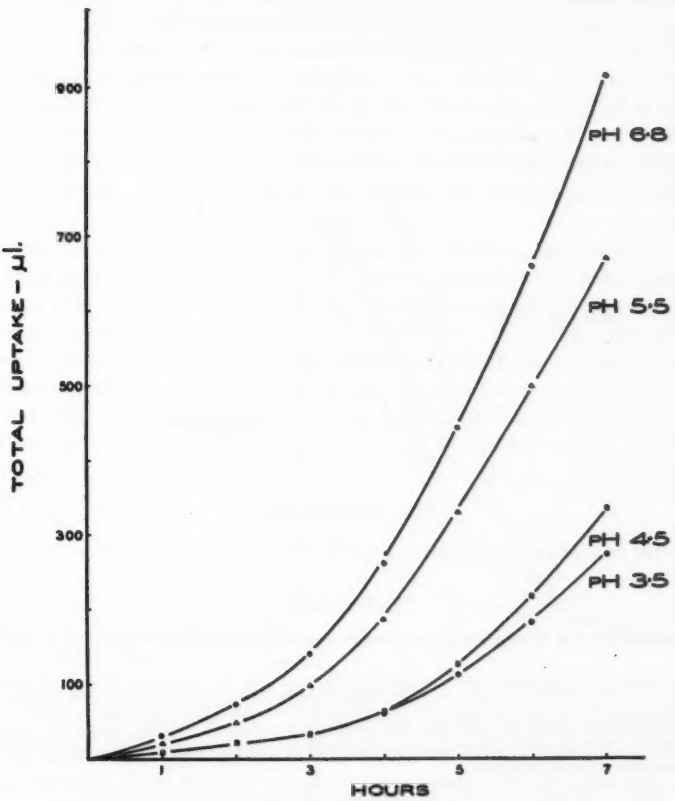

FIG. 7. Influence of soil reaction on oxidation of casamino acids. Vessels contained 4 gm. soil adjusted to pH indicated, 0.5 ml. casamino acids (10 mgm.), water to 1.5 ml., 0.2 ml. 20% KOH. Values for control (unamended) soils subtracted.

Oxidations with Different Soils

The soil used in the above experiments was a comparatively infertile greenhouse soil. Tests were conducted also with a variety of other soils with casamino acids as substrate. As may be seen in Fig. 8, the activity of the unamended soils varied markedly. Soil G, the poorest (greenhouse) soil, had the lowest respiratory activity. Soil N, an unfertilized field soil, somewhat more fertile than G, showed slightly greater activity but not as great as the same soil (X) which has received annual manurial applications. Soil BC is a rich compost soil and NG a soil with high organic matter content. In the presence of substrate a somewhat different picture appeared, except with soils G and N. Soil NG showed less activity than X and was considerably less active than BC.

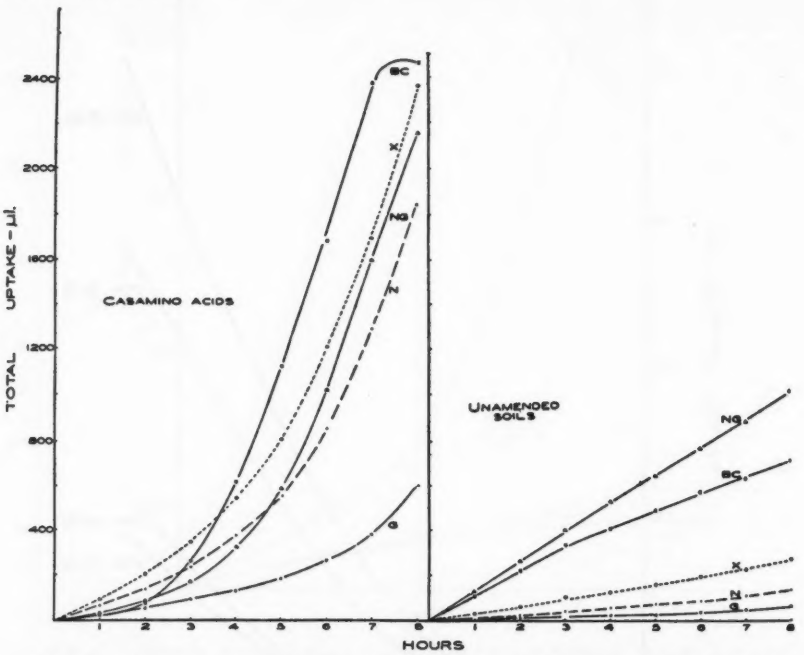

FIG. 8. Metabolic activity of different soils as influenced by casamino acids. Each vessel contained 4 gm. soil and 0.2 ml. 20% KOH. Unamended series: Soils N, X, G received 1.5 ml. water, BC and NG, 2.0 ml. (70% of moisture holding capacity). Casamino acid series: 10 mgm. in same volumes of water as used in unamended series; values for unamended soils subtracted.

Discussion

The foregoing studies indicate the possibility of carrying out manometric studies on soil simply and comparatively rapidly. The use of a suitable substrate may serve to emphasize the activity of the soil population by increasing the reaction rate. However, a fairly reliable indication of the metabolic

potential of a soil may also be obtained with the soil alone (Fig. 8). The respiratory activity of such unamended soil will reflect the amount and availability of oxidizable material in the soil, the activity of the population itself and possibly also the treatment it has received. The "autocthonous" flora of a virgin soil may show relatively low metabolic activity whereas a soil which has been cultivated, has received organic matter in various forms, and has a vigorous "zymogenic" population may be very active.

Another point of interest deriving from these experiments is the nature of the response obtained when a substrate such as casamino acids is added to soil. It is suggestive of a typical adaptive reaction, a conclusion which receives some support from the observed inhibition of oxygen uptake by 2,4-dinitrophenol and azide which are known as inhibitors of enzymatic adaptation. That this may represent an adaptive phenomenon is further suggested by the observation that there is no significant change in the number of organisms during the period of oxidation. It is possible, however, that this rapid increase in rate of oxygen uptake is due to the physiological activation of various microbiological elements in the soil (germination of spores of fungi, actinomycetes, and bacteria, etc.) followed by rapid assimilation and oxidation of the substrate. Further studies, including the use of other metabolic inhibitors, may shed light on this phenomenon.

It is clear that the manometric technique for studying respiratory activity in soil has potentialities which warrant further exploration. It holds particular interest as a rapid means of determining the resultant of all the associative and antagonistic forces which are operative in soil, thus permitting the study of soil as an ecological unit. It may facilitate investigations on the activity of various groups of soil organisms (by the use of selective antibiotics) and, when combined with suitable chemical and biochemical methods, may be a useful tool in studies on the nitrogen economy of soil (4), soil fertility, and related problems.

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